Compartment Specific Clone Generation

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To my family…

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ZUSAMMENFASSUNG

Kompartimente werden definiert als unterschiedliche Zellpopulationen, die aneinander grenzen. Stehen sie in direktem Kontakt, formen sie eine Grenze, die verhindert, dass sich Zellen aus verschiedenen Kompartimenten mischen. Die *Drosophila* Flügelimaginalscheibe ist in vier solche Kompartimente unterteilt: Anterior (A), Posterior (P), Dorsal (D) und Ventral (V). Folgendes „zentrales Dogma“ beschreibt die Entstehung der Kompartimentsgrenze: (i) Unterschiedliche Aktivität eines Selektorgens, wie zum Beispiel „engrailed“, zwischen zwei Kompartimenten gibt den Zellen innerhalb eines Kompartiments dieselbe Identität, (ii) die Spezialisierung sogenannter Grenzzellen wird durch Zell-Zellkommunikation über kurze Distanz zwischen Zellen benachbarter Kompartimente induziert und (iii) Grenzzellen geben weitreichende Signale in Form von Morphogenen ab, die das Wachstum und die Form des gesamten Flügels regulieren. Daher ist die Erhaltung einer klaren Grenze zwischen den Kompartimenten unabdingbar für die Erhaltung der Morphogenquelle, welche wiederum wichtig für das korrekte Wachstum und die richtige Form des Flügels ist.

Für die Integrität der A-P Kompartimentsgrenze ist die Hedgehog (Hh) Signalkaskade essentiell. Störungen in der Hh Signalkaskade führen zu Segregationsdefekten von Zellen an der Kompartimentsgrenze. Obwohl die Notwendigkeit dieses Signalwegs für die Kompartimentsgrenze bekannt ist und auch der physikalisch/mechanische Charakter der Kompartimentsgrenze intensiv studiert wurde, sind weder alle Zielgene der Hh Signalkaskade noch die Details der Segregation der Zellen bekannt. Dies liegt vor allem daran, dass die entsprechenden genetischen Werkzeuge, um diese Vorgänge zu studieren, bisher nicht vorhanden waren.

In dieser Arbeit beschreiben wir eine neue Methode, die wir „Generierung kompartiments-spezifischer Zellklone“ genannt haben. Die Methode besteht aus einer Kombination verschiedener genetischer Werkzeuge, wie Kompatiments-spezifische Treiber von LexA Transaktivatoren, $Gal80^{ts}$ und ein Gal80-unempfindliches Gal4 flip-out

System. Die Kombination dieser Werkzeuge ermöglicht uns, kompartiments-spezifische Klone in einer zeitlich regulierbaren Art und Weise, sowohl im A- als auch im P-Kompartiment, herzustellen. Die generierten Klone können mittels eines UAS-Konstrukts manipuliert werden, welches von einem konstitutiven Promotor kontrolliert wird. Somit wirken sich Manipulationen an der Aktivität des kompatiments-spezifischen Promotors nicht auf die Expression des UAS-Konstrukts aus. Mit dieser neuen Methode haben wir im A-Kompartiment Klone generiert, die eine reduzierte Hh Singalaktivität aufweisen. Diese Klone zeigen Segregationsdefekte - sie wandern in das P-Kompartiment ein. Dieser Ansatz ermöglicht das Screening von Targetgenen und die Beobachtung der Segregation in Echtzeit.

SUMMARY

Compartments can be defined as different, adjacent cell populations, which upon contiguity create a lineage boundary. This boundary prevents the movement of cells from different lineages across the barrier. The *Drosophila* wing disc is divided into four such compartments: anterior (A), posterior (P), dorsal (D) and ventral (V). The 'central dogma' outlining the main steps in the boundary formation and maintenance is: (i) a difference in the activity of a selector gene like, *engrailed* in the P compartment, gives an unique genetic identity to the cells within a compartment, (ii) short-range signaling between the cells of neighboring compartments establishes specialized border cells, (iii) finally border cells emit long-range signals, in form of morphogens, that regulate growth and patterning of the entire wing. The maintenance of the morphogen source is important for the correct morphogenesis of the tissue, which is done by maintaining a straight boundary between the two compartments.

The Hedgehog (Hh) signaling is essential for integrity of the A-P compartment boundary in the wing and the dysfunction leads to a segregation defect of cells at the boundary. Although the requirement of the pathway and the physical/mechanical nature of the boundary have been extensively studied, the target genes and the details of the segregation mechanism remain poorly understood. One reason for this is a lack of genetic tools to rigorously dissect the mechanism.

We developed a novel genetic technique that we named as the Compartment Specific Clone Generation (CSCG) technique. This technique utilizes a combination of several genetic tools, such as compartment-specific LexA transactivator drivers, $Gal80^{ts}$, and a Gal80-insensitive Gal4 flip-out system. Generating and validating appropriate LexA drivers was a major hurdle and represent a significant part of this thesis. The CSCG technique permits a temporal control of the compartment-specific clone generation. The clones can be manipulated by a UAS-construct that is driven by a constitutive promoter. Therefore, manipulations of the compartment specific promoter activity do not influence the UAS-construct expression. In proof of principle experiments we have used the

technique to generate clones with reduced Hh signaling activity in the A compartment. As expected, these clones showed the segregation defects – they moved into the P compartment. The validated and optimized approach enables conducting a genetic screen for the novel genes involved in boundary formation and more detailed live-imaging analyses of the segregation behaviors.

1. INTRODUCTION

1.1. Growth and Patterning in *Drosophila*

The body plan of an organism consists of patterns that are formed very early in development. This pattern formation is the outcome of self-organization of cell fates in space and time. Genes control the sequential yet complex organization of the pattern formation process. During patterning a 'selector gene' is expressed in one group of cells, but not in the other group. Cell-cell communication by signaling molecules between these two cell groups leads to the expression of long range signaling molecules, morphogens, in localized cell groups (Figure 1.1) that take care of the overall patterning and growth of the tissue (Dekanty and Milán, 2011; Zecca et al., 1995).

The selector gene expression assigns a unique identity to the cells. Distinct groups of cells, one with and the other without selector gene expression, are designated as 'compartments'. As visualized when examining the behavior of genetically marked clone of cells, cells of one compartment do not mix with the cells of the other compartment, thus indicating the presence of an invisible boundary, the compartment boundary (Bellido, 1973; Lawrence et al., 1996; Meinhardt, 1983).

Much of our understanding of the key concepts about how compartment boundaries arise comes from the experiments done on the *Drosophila* wing imaginal disc. *Drosophila* imaginal discs are tissue specific progenitors that later give rise to corresponding adult body parts. In the beginning the wing imaginal disc contains about 30 cells and this number reaches up to 50,000 cells during later larval stages (Milán et al., 1996). After metamorphosis the wing disc evaginates and forms an adult wing.

Compartments maintain a straight boundary despite the rearrangement caused by the cell division. The straight boundary is important, as the cells at the boundary are so called organizers, which are important for the secretion of morphogens and thus for providing

positional instructions during the growth of the tissue (Dahmann and Basler, 1999; Irvine and Rauskolb, 2001).

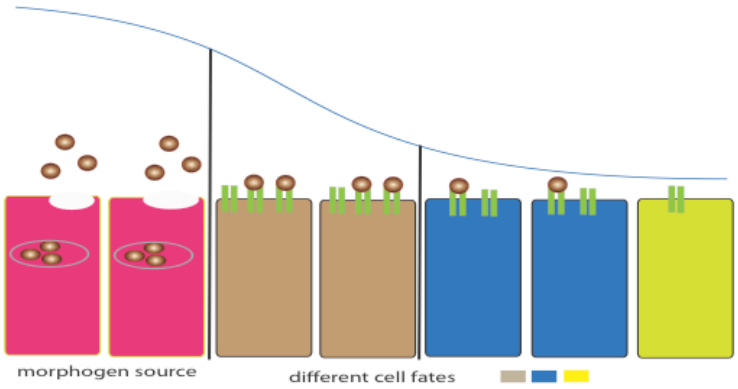


Fig 1.1: Illustration of patterning by morphogen gradient induced cell fates: morphogens (brown round structures) are synthesized at a localized morphogen source (pink rectangular structures showng cells). Morphogens disperse from production site and bind to cell receptors (two green lines) along the way, resulting in spatial concentration gradient of morphogen-receptor complexes. Differential cell signaling gives rise to different gene expressions (denoted by different color rectangular cells) giving rise to patterns.

1.2. Compartment Boundaries of the *Drosophila* Wing Imaginal Disc

The wing imaginal disc of *Drosophila* provides an excellent model for the study of compartments. Much of our understanding of the key concepts about compartment boundaries comes from the experiments done in the *Drosophila* wing disc. Due to its simple structure and accessibility to genetic manipulations wing discs of *Drosophila* are widely used as a model system to study growth and patterning. Broad relevance of the results shown on *Drosophila* wing discs is found in both invertebrates and vertebrates.

The wing disc is divided into dorsal-ventral (D-V) and anterior-posterior (A-P) compartments. The key features of and differences between the D-V and the A-P boundary are listed below:

1.2.1. The Dorsal-Ventral Compartment Boundary

The boundary subdividing a wing disc along the dorsal-ventral axis originates during the second instar stage of the larvae. Clone of cells in the dorsal compartment that lack *apterous (ap),* a LIM/homeobox gene, function show a cell autonomous transformation to a ventral identity (Diaz-Benjumea and Cohen, 1993).

ap drives the expression of *fringe*, a glycosyltransferase, in the dorsal cells (Irvine and Wieschaus, 1994). Via its glycosyltransferase activity Fringe modulates the binding of Notch to its two ligands, Serrate and Delta (Brückner et al., 2000). Glycosylated Notch preferentially binds to Delta but not Serrate. On the other hand, the unglycosylated Notch receptor binds to Serrate but not to Delta. Consequently, signaling by each ligand is limited to nearby cells on the opposite sides of the boundary, with the result that high levels of Notch activity are limited to a narrow band of cells along the D-V boundary (Becam and Milán, 2008). Upon binding to the ligand the intracellular domain of Notch

is cleaved and translocates into the nucleus where it induces the expression of the target genes, *vestigial (vg)* (Williams et al., 1994) and *wingless (wg)* (Diaz-Benjumea and Cohen, 1995). It has been shown that the Notch signaling coordinates tissue growth and wing fate specification in *Drosophila* (Rafel and Milán, 2008).

Although altering the signaling properties of cells by modulation of *fringe* activity has been shown to allow cells to cross the boundary (Rauskolb et al., 1999), *fringe* activity has been shown to be insufficient to support boundary formation (Milán and Cohen, 1999). On the other hand results show *ap*, *capricious (caps)* and *tartan* together contribute to the D-V boundary formation in the wing disc (Milán et al., 2001). *Caps* and *tartan* encode transmembrane proteins with extracellular leucine-rich repeats (LRR) that are expressed in the dorsal cells and have complementary roles in boundary formation, together with *fringe*.

The interface between *apterous*-expressing and non-expressing cells is displaced from the line, at which *Notch* is activated (Rauskolb et al., 1999). Notch does not make a compartment boundary by allotting specific dorsal-type or ventral-type cell affinity. On disruption of Notch signaling cells can intermix on either side. Uncoupling Notch signaling from the requirement for the Apterous activity by expressing Serrate or Fringe in the dorsal cells in an *apterous* mutant disc results in induction of the D-V organizer. However, Apterous activity is insufficient to specify dorsal cell fate and the resulting wing adopts the ventral identity on both the surfaces. The boundary between the D and V compartments is disrupted in these conditions, indicating that the Notch signaling is not sufficient to induce the D-V affinity boundary. Hence, another Apterous-dependent mechanism is likely to prevent the mixing of the dorsal and ventral cells. Transient expression of Caps and Tartan, contributes to the formation of the affinity boundary. Apterous controls the expression of Serrate and Fringe as well as Caps and Tartan in the dorsal cells during the boundary formation (Milán, M. and Cohen, S.M., 2003). On the other hand a permissive role of Notch has been shown in maintaining the D-V affinity

boundary (Becam and Milán, 2008). Still a deeper understanding of the process by which these proteins interact and boundary formation takes place remains elusive.

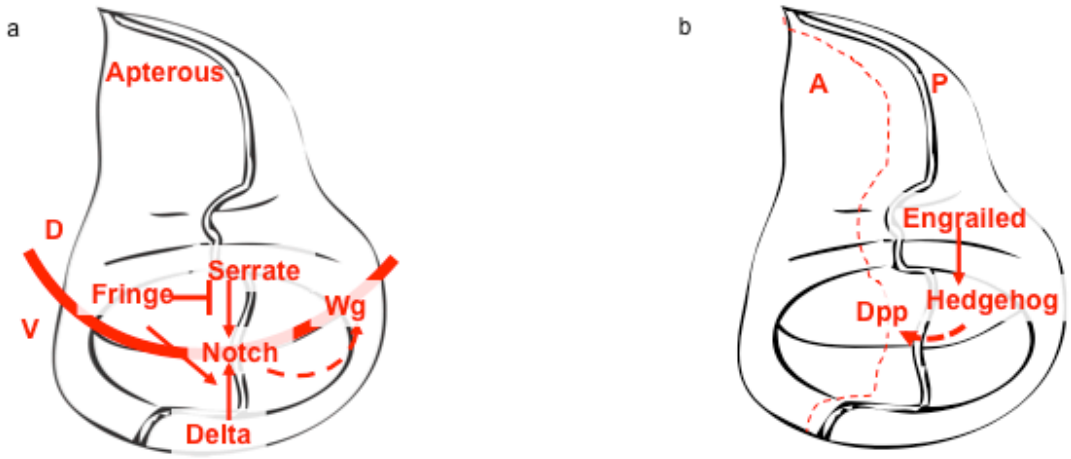


Fig 1.1: *Drosophila* wing imaginal disc compartments – (a) *apterous* is expressed in the dorsal (D) cells and induces *fringe* expression in D-cells. Fringe glycosylates Notch, glycosylated Notch preferentially binds to Delta but not Serrate. Aglycosylated Notch binds to Serrate. *Wingless (wg)* is expressed in a narrow band of cells at the D-V boundary (shown in red thick line). (b) *engrailed (en)* is expressed in the posterior (P) cells and induces expression of *hedgehog (hh)*. Hh travels to a short distance into the anterior (A) compartment and induces target gene like, *decapentapegic (dpp)*, in a thin stripe of cells along the A-P boundary (for details of Hh signaling see Fig. 1.2).

Important observations have been made that in wing discs filamentous (F)-actin and non-muscle Myosin-II are enriched at adherens junctions along the D-V boundary (Major and Irvine, 2005, 2006). These observations indicate a role of Notch in actin organization at the D-V boundary. Moreover Myosin-II localization and requirement has been shown at the D-V boundary. These findings lead to a suggestion that Myosin-II generated high tension along the boundary can be a consequence of both biochemical and mechanical mechanisms. One mentionable work in this line is a gain-of-function suppressor screen for genes involved in D-V boundary in the *Drosophila* wing (Bejarano et al., 2008). In future it will be interesting to investigate the nature of these mechanisms.

1.2.2. The Anterior-Posterior Compartment Boundary

First Morata and Lawrence (1975) showed that the posterior cells homozygously mutant for the *engrailed (en)* gene, a homeodomain transcription factor, fail to respect the boundary. Hence *en* is the marker of 'posteriorness'. Expression of *en* establishes the A-P boundary very early in the development. When discs are specified in the embryo, small groups of *en*-expressing and non-*en*-expressing cells are formed (Cohen et al., 1993). Separation between these two groups of cells is maintained throughout the course of embryogenesis (Morata and Lawrence, 1975). Posterior *engrailed* expressing cells induce *hedgehog (hh)* expression (Figure 1.1b). Since Hh is a short-range secretory signaling molecule, only a narrow stripe of A-cells along the boundary can respond to the Hh signaling in a unidirectional manner. In contrast to Notch signaling at the D-V boundary, the signaling at the A-P boundary is asymmetrical.

1.2.2.1. Hedgehog Signaling

Hh is produced in the posterior but not in the anterior compartment of the wing disc. Hh induces its signaling to the first few rows of cells anterior to the compartment boundary (Méthot and Basler, 1999). In these cells, Hh directs the transcription of target genes such as *decapentaplegic (dpp)* and *patched (ptc)*.

The Hh protein is a secreted molecule, synthesized as a precursor of 400-500aa length. The precursors undergo modification to give rise to the active signaling ligand. P-cells are refractory to Hh signaling, whereas A-cells can respond to it. Two membrane proteins have been implicated in the reception and transduction of the Hh signal-transduction pathway, Patched (Ptc), a multiple-pass transmembrane protein (Hooper and Scott, 1989; Nakano et. al., 1989) and Smoothened (Smo), a seven-pass transmembrane protein (Figure 1.2). Ptc functions as an inhibitor of Hh signaling and limits the Hh signaling in its range (Chen and Struhl, 1996).

Several proteins have been identified as components of Hh signaling like, Costal-2 (Cos-2), Protein Kinase A (PKA), Suppressor of fused (Su (fu), Slimb and Cubitus interruptus (Ci). The zinc finger transcription factor, Ci (Méthot and Basler, 1999; Dominguez et al., 1996) is a key component of the downstream signaling. Ci is a large protein that can be cleaved into a repressor form Ci^{rep} or can activate the target genes as a full-length activator form Ci^{act} (Chen et al., 1999b).

In absence of Hh (Figure 1.2) Ptc inhibits Smo activity. PKA phosphorylates the C-terminal region of Ci and processes Ci in a way that inhibits its transcriptional activity (Chen et al., 1999; Wang and Holmgren, 1999). Slimb acts downstream of PKA and targets phosphorylated Ci for ubiquitin-mediated proteolysis (Wang and Holmgren, 1999, Jiang and Struhl, 1998). Proteolysis of Ci produces Ci^{rep} that represses the Hh target gene expression in the nucleus.

The Hh-activated Ptc does not repress Smo. Smo can now interact with Cos-2, a kinesin-related protein, to change its high affinity association with microtubules (Robbins et al., 1997). In turn this inhibits Ci by tethering it in the cytoplasm. Ci is found in a tetrameric complex together with Fu, Su(Fu) and Cos-2 (Robbins et al, 1997, Sisson et.al., 1997, Monnier et al, 1998, Stegman et.al., 2000). This complex formation tethers Ci to the cytoplasm and locates it to the site of Slimb-dependent proteolytic processing. Hh signaling alters the complex to release from microtubules, resulting in the formation of Ci^{act}. Fu gets stimulated by Hh signaling and counteracts the inhibition of Su(fu) on Ci^{act} and accumulates Ci^{act} in the nucleus (Méthot and Basler, 2000). In the nucleus Ci^{act} activates Hh target genes.

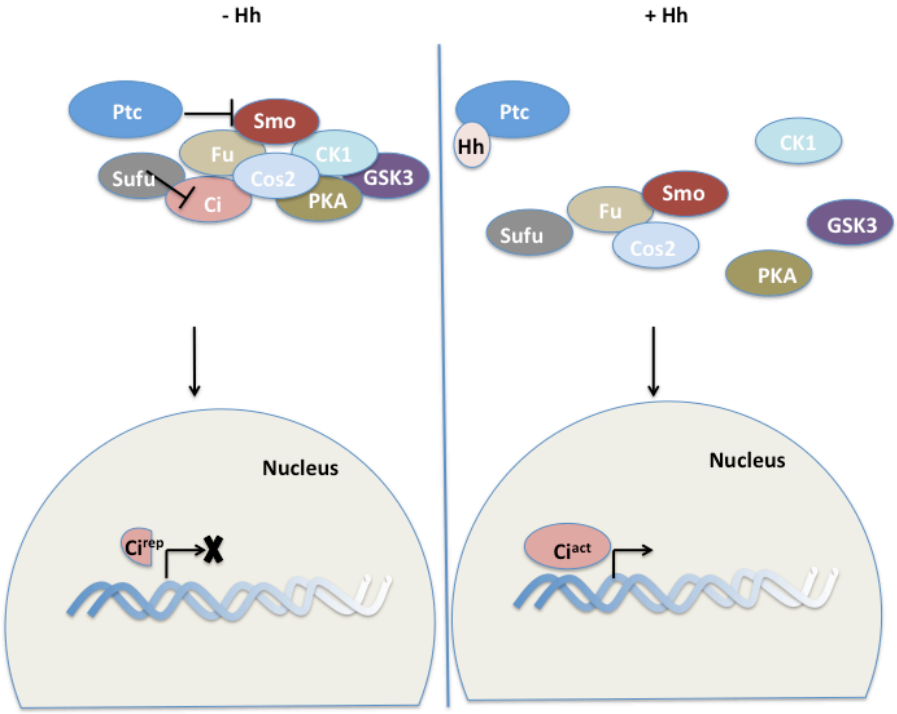


Fig 1.2: Hedgehog signaling pathway – In absence of Hedgehog (Hh) ligand (left panel) Patched (Ptc) prevents the activity of Smoothened (Smo). Cubitus interruptus (Ci) forms a complex with Costal-2 (Cos-2) and is localized in the cytoplasm. Phosphorylation of Ci by protein kinases PKA, GSK3 and CK1 leads to Ci proteolysis that generates a shorter form of Ci repressor form Ci^{rep} . Ci^{rep} represses Hh target genes in the nucleus. In presence of Hh (right panel) the intact Ci^{act} protein levels Ci^{rep} and induces Hh target gene expression in the nucleus like *dpp*. (Figure modified from www.abcam.com)

1.2.3. Differences Between the A-P and the D-V Boundary Formation Mechanisms

Lineage restrictions in the A-P and the D-V compartments possess some common features; but there are some differences between them. The fundamental difference between the mechanism of the A-P and the D-V boundary formation is that the signaling across the D-V boundary is symmetric but in A-P boundary it is not. Short-range signaling between D and V cells result in symmetrical activation of Notch signal transduction (Figure 1.1) on both sides of the compartment boundary (Diaz-Benjumea and Cohen, 1993; Kim et al., 1995). Moreover, unlike the A-P boundary, the D-V boundary is congruent with the wing margin. In the late larval development this region is observed to have reduced cell division rates (O'Brochta and Bryant, 1985). Nevertheless the D-V boundary formation mechanism also strongly supports the selector gene hypothesis: loss of *apterous (ap)* function from dorsal cells causes them to cross into the ventral compartment (Blair et al., 1994). Hence localized expression of *ap* specifies dorsal cell fate (Cohen et al., 1992; Williams et al., 1993) .

1.3. How is the A-P Boundary Maintained?

Investigations in the past showed that *engrailed* plays a key role in distinguishing the posterior and the anterior cells. On the other hand *engrailed* influences the cells of the anterior compartment by Hh signaling. Hh-mediated signaling between A and P cells plays a major role in creating the differences at the compartment boundary (Rodriguez and Basler, 1997). Based on these observations the following hypotheses have been put forward to explain the process of boundary maintenance:

1.3.1. The Differential Cell Affinity Hypothesis

Tissue separation could be driven by differences in the quantity and quality (Figure 1.3) of the adhesive interactions among cells (Steinberg, 1955). Steinberg modeled that depending upon the relative strengths of adhesive interactions between and among different cell types the mixture of cells may intermix, envelope, adhere yet remain in distinct phases, or separate.

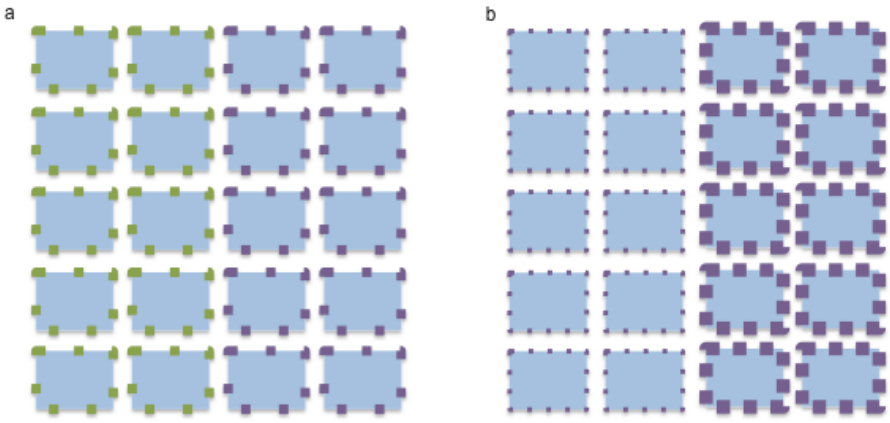


Fig 1.3: Differential cell affinity hypothesis – (a) cell segregation can take place because they express different kinds of cell adhesion molecules (shown by green and blue dots). (b) cell segregation can take place because they express different levels of the same cell adhesion molecule (shown by blue dots).

Hh signaling transcriptional outputs have been shown to play a key role in specifying the "A-type" cell sorting behavior (Dahmann and Basler, 2000). Hh non-transducing A-cells do not respect the boundary and "sort out" into the P compartment. However, we still lack clear evidence that cell adhesion is the underlying mechanism for sorting of cells lacking the Hh signaling. Alteration of the cell adhesion properties could include modulation of the adhesion complex by interaction with the actin cytoskeleton. Hence it is possible that the Hh signaling affects cell sorting by modulating small GTPases.

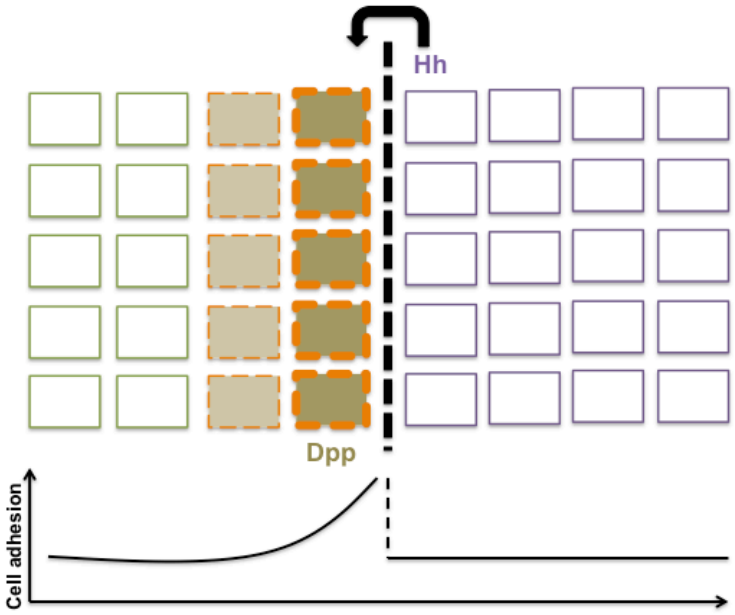


Fig 1.4: Hedgehog signaling induced change in affinity is responsible for cell segregation – abrupt change in the levels (or quantity) of adhesiveness (shown with orange dots) in the A-cells that are next to the boundary result in segregation of A and P cells. (Figure modified from Dahmann and Basler, 2000).

1.3.2. The Increased Cell Bond Tension Hypothesis

Recent studies have shown that also physical forces have a pivotal role in the boundary maintenance. *zipper* encodes Myosin II heavy chain. In *zipper* mutant wing discs the roughness of the compartment boundary is greater than the compartment boundary in the wild type wing discs (Landsberg et al., 2009). On application of an inhibitor for the Rho-kinase, whose main effector is Myosin II, the straightness of the boundary is also compromised (Landsberg et al., 2009). These results indicate a role of the actomyosin enrichment in the border cells for maintaining the boundary. It has been shown that a 2.5 fold increased cell bond tension is sufficient to maintain the compartment boundaries (Landsberg et al., 2009).

1.4. Objectives and Scope

Despite the identification of borders and the genes responsible for establishing them, a detailed understanding of the molecular mechanisms that actually keep cells separated is lacking. The goal of the experiments described in this thesis is to generate the tools to better understand the mechanisms of the compartment boundary formation.

Until now the best set of experiments showing involvement of various Hh signaling components in the boundary maintenance have been designed around 'traditional' genetic tools like *FLP/FRT* systems. As a result either genetic mosaics are formed that involve generation of homozygous mutant cells from heterozygous forebear or ectopic expression of transgenes in clones take place by the *act>stop>Gal4* and *FLP* system. One example of such an attempt is a screen based on the *FRT/FLP* system (Végh, M and Basler, K 2003). In this approach double *FRT* chromosomes in combination with a wing-specific *FLP* source were used to screen mutagenized chromosomes, this is a very complicated and time consuming process.

In this thesis we describe the generation of a novel technique that temporally and spatially controls clone generation. On the other hand the generated technique makes it simple to modulate the genetic dosage in a clone by integrating *UAS-RNAi* mediated knock down. The two features of the technique, which we call the Compartment Specific Clone Generation (CSCG) technique, are:
1. Precise: we should be able to generate clones exclusively in one compartment of the wing disc.
2. Convenient: ability to directly engage the *UAS-RNAi* library in a one step-crossing scheme.

The technique is a combination of several different genetic tools including two binary transcriptional systems: the *Gal4/UAS* and the *LexA/lexO* system. The *LexA/lexO* system (Figure 2.1a) is based on a bacterial transcription factor LexA that binds specifically to

the *lexA Operator (lexO)* enhancer to activate a targeted gene expression (Lai and Lee, 2006; Yagi et al., 2010). The *LexA*/*lexO* system functions independent of the *Gal4*/*UAS* system. We selected the *LexA*/*lexO* system to first start the chain of genetic events by producing the *FLP* recombinase in a compartment specific manner. Later the well-regulated series of genetic conversations between the components of the developed technique will produce Gal4 expressing clones in a specific compartment of the wing disc (see Figure 2.3.1a). In the interest of using the *UAS-RNAi* overexpression to knockdown the desired gene in the clone it was necessary to express *Gal4* in the clone.

The generation and validation of CSCG technique involved the following steps:
(1) Enhancer trap screen to find genes that are expressed at the A-P boundary and may play a role in the boundary maintenance.
(2) We constructed a P-compartment specific LexA TA by adapting 'homing'.
(3) We generated two separate A and P compartment specific clone generation systems and proved the functionality of the systems.
(4) We compared the efficiency of the already existing clone generation system with our CSCG technique.
(4) We established the CSCG technique further by conducting a pilot screen for the genes involved in the boundary maintenance.
(5) We studied the border cell morphology with the help of CSCG technique.

2. RESULTS & DISCUSSION

2.1. Enhancer Trap Screen

The primary key component needed for the CSCG technique is a compartment-specific LexA-transactivator (LexA TA) driver (see section 2.3 and figure 2.3.1a). To obtain such a LexA TA driver we performed an extensive screening to identify a compartment-specific G-MARET (Gal4-based Mosaic-inducible And Reporter-exchangeable Enhancer Trap) insertion. The G-MARET is a *P*-element enhancer trap system recently developed in our lab. The system permits exchange of the *Gal4* in the *P*-element insertion, $P\{\geq Gal4,w^+\}^{mr}$ (also described as *mrN≥Gal4* in which N indicates an insertion clone number) with another reporter gene, such as a *LexA TA*. As a result we can readily generate a $P\{\geq LexA\ TA,w^+\}^{mr}$ without changing the expression patterns of the original *mrN≥Gal4* insertion (Yagi et al., 2010). The $P\{\geq Gal4,w^+\}^{mr}$ is expected to be efficiently mobilized on chromosomes and thus can produce new insertions efficiently. The $P\{\geq Gal4,w^+\}^{mr}$ is a derivative of *P{GalW},* a P element vector with a high mobilization efficiency that was generated in out group (Gerlitz et al., 2002).

We conducted a pilot screen to find out: (i) which $\{\geq Gal4\}^{mr}$ insertion line is suitable to use as a starter line (ii) mobilization frequency of $\{\geq Gal4\}^{mr}$ and (iii) compare F1 vs. F2 screening method. X-chromosomal *pMaret-Gal4* insertion, *mr25≥Gal4* (clone #25; ≥ indicate a *loxP* site) was used for a pilot screening. Virgin females of the *mr25≥Gal4* carrying a $P\{\geq Gal4,w^+\}^{mr}$ insertion were crossed to the *Δ*2-3 *TM3 Sb* males. The F1 male progenies with mosaic red eyes due to both the *Δ2-3 TM3 Sb* and the $P\{\geq Gal4,w^+\}^{mr}$, were collected. These males are referred to as "jumpstarters", in which the $P\{\geq Gal4,w^+\}^{mr}$ is being mobilized in the germ line. Such jumpstarter males were crossed to *yw* virgin females. Jumpstarter males have the $P\{\geq Gal4,w^+\}^{mr}$ on the X chromosome hence F_2 male progenies with red eyes have a new autosomal or Y-chromosomal insertion of the

P{≥Gal4,w⁺}^mr. The above F_2 screening method is a sure way to isolate every new insertion. Nevertheless, pre-meiotic transposition events can lead to repetitive insertions therefore only one resulting transposant from each jumpstarter cross was used to establish a new line. The number of individual jumpstarter fly crosses determines the maximal number of transposants recovered. It is likely that not every cross with a single jumpstarter yielded a new insertion.

We calculated the mobilization frequency for the starter line *mr25≥Gal4:* we calculated the ratio of the number of the F_2 males with w^+ Sb^+ per that of the total F_2 males. For this purpose we made 20 individual crosses of one jumpstarter male with several *yw* virgin females and counted number of the F_2 males with distinct genotypes. The transposition frequency was 5.5% that is much less than for *pGalW*, which was 10% (Gerlitz et al., 2002). Hence, we conclude that the F_2 screening with the *mr25≥Gal4* was not efficient for a large-scale screening.

Therefore we switched to a *GFP*-based F1 Screening method that was previously developed in our group (Gerlitz et al., 2002). In the F_2 screening, we needed to isolate F_2 flies with a new insertion and observe *Gal4* expression patterns of a new insertion in the F_3 generation. By contrast, the F_1 Screening method uses the jumpstarters to observe *Gal4* expression patterns of the F_2 larvae by crossing to a *UAS-GFP* line. The larvae is semi-transparent that allows observing *GFP* expression patterns in wandering 3rd instar larvae under a UV stereomicroscope. Thus, although the F_2 generation is a collection of the animals with different genotypes – e.g. no, single or multiple *P{≥Gal4,w⁺}^mr* insertions with or without Δ2-3) – we can readily isolate larvae with interesting patterns of *Gal4>GFP* expression (i.e. a compartment-specific pattern).

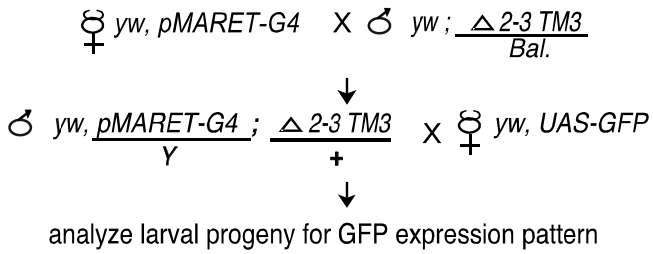


Fig. 2.1b: Enhancer trap screen crossing scheme

We conducted a large-scale screen by using the *GFP*-based F_1 screening method and the *mr25≥Gal4*. A total of ~200 bottles were set up, each containing 5-7 jumpstarter males crossed to ~30 homozygous *UAS-GFP* virgins females. Two to three transparencies were screened per bottle, thus a total of ca. 500 transparencies were screened. On average each transparency contained 100-150 third instar larvae, of which 50% contained the heterozygous *P{≥Gal4,w⁺}^mr* element. Of ~22,000 new *P{≥Gal4,w⁺}^mr* insertions screened, 15 lines were established, which show a spatially restricted expression pattern in the wing disc. Low survival under the UV and failure to establish lines from larvae that also possess transposase chromosome attributed to the low number of lines finally established. Also it has been reported previously that there are some sites where P elements insert preferentially (Liao et al., 2000; Tower et al., 1993). For this reason we could see some repetitive expression patterns which also decreased the total number of new isolated lines. These were the reasons that made us assume that with this method the chances of getting a compartment-specific insertion were quite low and would be time consuming.

Before establishing each fly line, we isolated twice the insertion showing the *Gal4* expression patterns from any other insertion. Since the *UAS-GFP* line does not carry w^+ but carries a *yellow* (y^+), we could choose the larvae by tracing the expression patterns and the fly with reduced w^+ activity after the recombination. To map the site of insertion, we performed inverse PCR (iPCR) (Ochman et al., 1988). For most of the established lines the integration site could be determined (Figure 2.1c).

2.1.1. The Newly Obtained G-MARET Insertions Show Various Patterns of *Gal4* Expression in the Wing Disc

The *mr≥Gal4* lines we newly established and the *Gal4* expression patterns in wing disc are summarized in Figure 2.1c.

Besides the lines with a compartment-specific expression, we chose to also establish the lines with interesting expression patterns, such as pouch-specific, and hinge-specific. Interestingly many insertions that showed familiar expression patterns did not correspond to the respective known genes like, F2-IG-2010-1 and F2-IG-815 (Figure 2.1c).

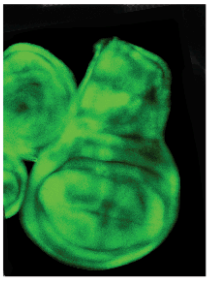
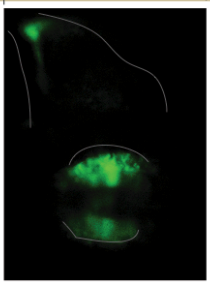
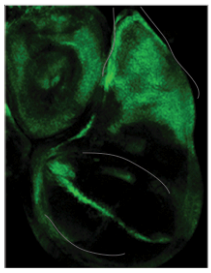
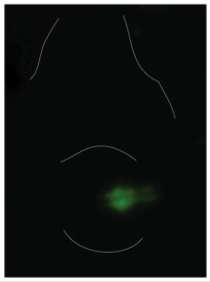
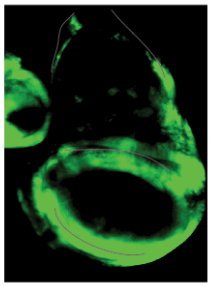
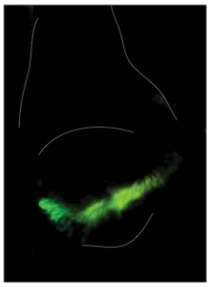
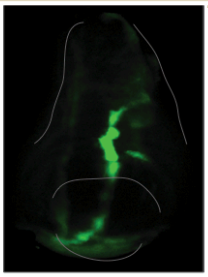
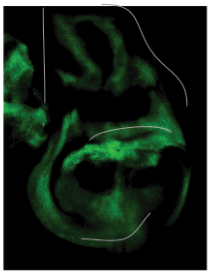
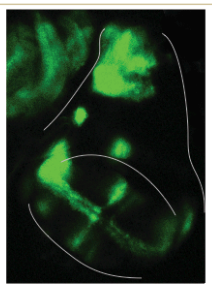
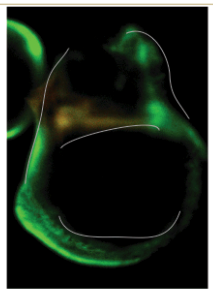
Insertion	Description	Insertion	Description
	Insertion: F2-IG-232 Known gene: NA Chromosome: II iPCR: NA Expression: ubiquitous		Insertion: F2-IG-226 Known gene: klu Chromosome: 3L iPCR: 68A1-68A1 Expression: pouch and notum
	Insertion: F1-IG-23 Known gene: NA Chromosome: III iPCR: NA Expression: DV boundary, notum		Insertion: F2-IG-55 Known gene: NA Chromosome: III iPCR: NA Expression: DV border
	Insertion: F2-IG-19 Known gene: NA Chromosome: III iPCR: NA Expression: around pouch and notum		Insertion: F2-IG-103 Known gene: nmda1 Chromosome: 2R iPCR: 49D6-49D6 Expression: DV border
	Insertion: F2-IG-2010-1 Known gene: CG42669 Chromosome: 3L iPCR: 62E2-62E2 Expression: AP border		Insertion: F1-IG-09 Known gene: NA Chromosome: II iPCR: NA Expression: pouch, hinge and notum
	Insertion: F2-IG-815 Known gene: jim (CG11352) Chromosome: 3L iPCR: 80A2-80A4 Expression: pouch and notum		Insertion: F2-IG-63 Known gene: CG31612 Chromosome: 2L iPCR: 40A2-40A3 Expression: around pouch and notum

Fig. 2.1c: New mr≥Gal4 lines obtained from the screening – *Gal4* expression patterns in the 3rd instar wing imaginal disc. Gal4 expression was monitored by UAS-GFP expression. NA, not analyzed.

2.1.2. Confirmation of the Reporter-Exchangeable Function of the *mr232≥Gal4*

As mentioned above, the G-MARET system is designed for the subsequent unidirectional introduction of a second reporter (e.g., a *LexA TA*) with y^+ and a *loxP* site downstream of the *Gal4* (see Yagi et. al., 2010). This results in release of a *loxP* cassette with the *Gal4* and y^+, followed by the second reporter gene. Removal of the *loxP* cassette, by Cre recombinase, results either in a complete reporter exchange or a genetic mosaic with mutually exclusive *Gal4* or the second reporter gene expressing clones. Therefore it makes it possible to manipulate two adjacent cell populations independently.

To test whether the newly obtained *mr≥Gal4* lines have the reporter-exchangeable function, we chose *mr232≥Gal4*. *mr232≥Gal4* shows almost uniform expression of *Gal4* in the wing disc, which makes it useful for various experiments. A second reporter vector containing the *LHG* coding region, a *loxP* site, the y^+ marker, and an *attB* sequence was successfully integrated into the *attP* within the *pMARET-G4* (done by Dr. R. Yagi and F. Mayer). Such transformed individuals carry *mr232≥Gal4*,y^+*≥LHG*. When crossed to *hsp70-cre* line we could get a mosaic pattern of mutually exclusive *Gal4* and *LHG* positive cells, monitored by *UAS-CD8::GFP* and *lexO-mCherry::CAAX*, respectively (Figure 2.1d). The loxP cassette was "popped-out" spontaneously without a heat shock. This result indicates that at least one of the newly obtained mr≥Gal4 lines possesses the reporter-exchangeable function.

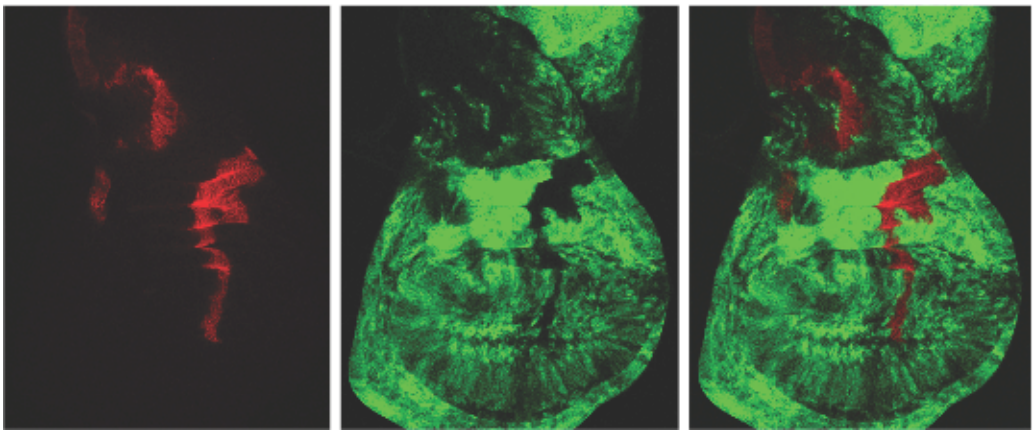


Fig. 2.1d: Wing disc with mosaic of *Gal4* and *LHG* expressing clones – *LHG* driven *lexO-mCherry::CAAX* (left), *Gal4* driven *UAS-CD8::GFP* (middle), mosaic of *Gal4* and *LHG* expressing clones (middle).

2.1.3. The A-P Boundary Specific *mr2010≥Gal4*

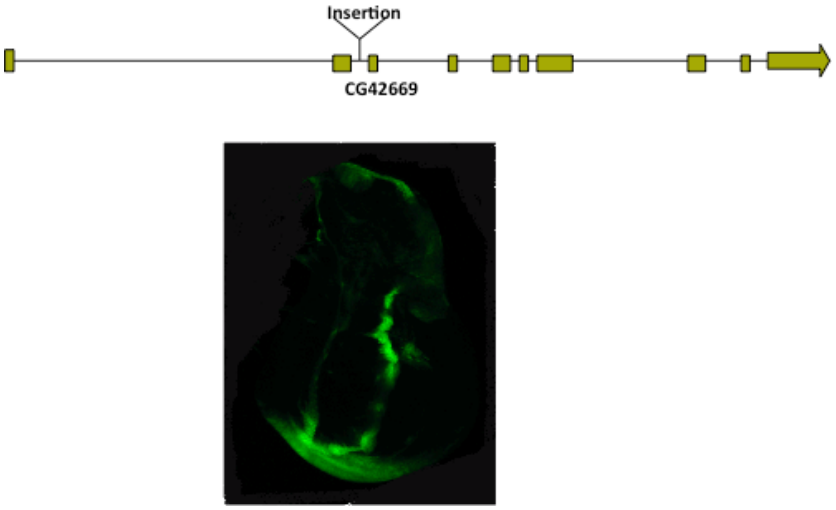


Fig 2.1e: *mr2010≥Gal4* is expressed at the A-P boundary – insertion mapped into the second intron of *CG42669*.

Among the newly obtained *mr≥Gal4* lines, *mr2010≥Gal4* alone shows a *Gal4*-expression pattern related to the compartment boundary (Figure 2.1e). *Gal4* is expressed in a stripe along the A-P boundary, which is reminiscent of Hh target genes such as *dpp* and *ptc* in wing disc.

We performed iPCR to map the insertion: it locates into the second intron of the *CG42669* gene. The features and predicted functions for the gene are unknown. Previously, Dr. G. Reim conducted a screen for new Hh target genes and CG42669 was a candidate found by microarray. She analyzed expression patterns of the gene by in-situ-hybridization (unpublished result; data not shown). The transcript does not show A-P boundary-specific patterns, unlike the *mr2010≥Gal4* expression pattern. The intronic region of *CG42669* may have a regulatory sequence that influences a neighboring transgene, but not the *CG42669* itself.

If the *CG42669* gene plays a role in the Hh signaling or in the maintenance of the compartment boundary, the cell clones with gain or reduced activity of the gene should behave abnormal at the boundary. To examine if manipulation of the gene activity induces cell-sorting behaviors, we generated *Gal4* flp-out clones, which express either *UAS-CG42669*^{RNAi} or *UAS-CG42669* both clones seem to behave normally and respect the compartment boundary, and also in adult flies no obvious phenotype could be observed. Therefore, it is unlikely that the *CG42669* gene plays a role in the A-P compartment boundary and the Hh signaling.

2.1.4. Conclusion

The enhancer trap screen gave us a diverse collection of G-MARET insertion lines. Although we did not get any insertion that was expressed in a compartment-specific manner, the obtained wing-specific G-MARET lines express *Gal4* in different patterns in the wing imaginal disc. The ability to convert the *Gal4* of G-MARET to a second reporter permits functional analyses of some complex biological phenomena, such as cell competition and regeneration. Such mosaics allow us to study situations where cell-cell communication between two adjacent cell populations play a pivotal role.

2.2. Generation of a P-compartment Specific LexA-TA by 'Homing'

To get the desired P-compartment specific LexA-TA expression we chose *engrailed* as a driver because *engrailed* is expressed in the posterior compartment of the wing disc (Morata and Lawrence, 1975). To do so we made use of the 'homing' phenomenon (Kassis et al., 1992). It has been shown that the P-constructs containing DNA fragment including the *engrailed* promoter and a 2.4 kb upstream sequence (*en* homing sequence) cause integration into the *en* region of the chromosome (Hama et al., 1990; Kassis et al., 1992). We designed a P element plasmid, *en-LHG-IG*, that contains the *en* homing sequence (Kwon et.al., 2009) and a LexA-TA (Yagi et al., 2010).

Introduction into flies was carried out with the P-element vector, the *en-LHG-IG* and LexA expression was monitored with the *lexO-rCD2::GFP* expression. The pattern accurately mimics the normal developmental profile of the *en* gene. The GFP expression in *en-LHG-IG*/+ animals at third-instar stage was found in segmentally reiterated stripes. The posterior compartment of all imaginal discs contained *en-LHG-IG* driven *lexO-rCD2::GFP* expression (Figure 2.2). GFP expression was consistent with the known patterns of *engrailed* expression. We also checked if the expression is posterior-specific and does not exhibit the 'Blair expression' (Blair, 1992) of *engrailed* in the anterior cells. We compared this expression of *en-LHG-IG* with an enhancer trap *en-LacZ* line, K537. Line K537 shows 'Blair effect free' *engrailed* expression. The expression of *en-LHG-IG* was confined to the posterior compartment of the wing disc even in late third instar stage. Further cytological location of the integrated plasmid was determined as 48A, in *en* locus, by performing inverse PCR (iPCR) (Ochman et al., 1988).

Previous studies show that the P elements transposition frequency to particular loci varies from frequencies of $<10^{-6}$ to 10^{-2} (Engels, 1983). Among ~2400 injected embryos ~1900 hatched and 6 showed GFP expression. Out of these 6 transformants, 2 showed posterior compartment specific expression. Based on the number of recombinants obtained that

showed expression in the posterior compartment, we find 'homing' to be an efficient way to integrate the DNA of interest into the *engrailed* locus.

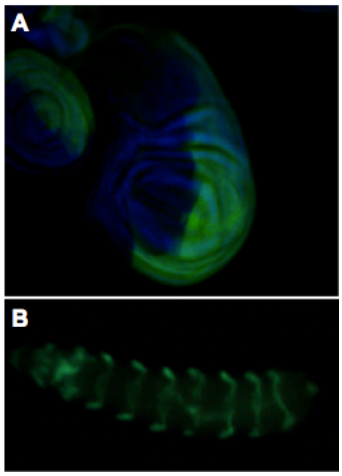


Fig 2.2: Expression of *en-LHG-IG* – (A) expression of *en-LHG-IG* driven *lexO-GFP* in posterior compartment of the wing disc. (B) in stripped pattern on larval body.

2.3. Compartment Specific Clone Generation (CSCG)

This section is written in the format of a Manuscript in preparation.

Summary

In Drosophila, the Hedgehog (Hh) pathway is essential for the integrity of the anterior (A)-posterior (P) compartment boundary in the wing. Dysfunction of the Hh signaling pathway leads to a segregation defect of the cells at the boundary. Although the requirement of the pathway and the physical/mechanical nature of the boundary have been extensively studied, the target genes and the details of the segregation mechanism remain poorly understood. Here we describe a novel technique that we named as the Compartment Specific Clone Generation (CSCG) technique. The CSCG technique utilizes a combination of several genetic tools, such as compartment-specific LexA transactivator drivers, $Gal80^{ts}$, and a Gal80-insensitive Gal4 flip-out system. The combination permits temporal and compartment-specific clone generation in either the A or the P compartment. The clones can be manipulated by a *UAS*-construct; manipulations on the compartment specific promoter activity do not influence the *UAS*-construct expression. As a proof of principle, using this technique, we generated clones with reduced Hh signaling activity in the A compartment. These clones showed segregation defect – they moved into the P compartment. The CSCG technique represents a powerful tool to conduct genetic screens for the genes involved in the compartment boundary formation or live-imaging analyses of the segregation behavior.

Introduction

The boundaries between juxtaposed gene expression domains prevent the two cell populations from intermingling with each other (Lawrence, 1977; Meinhardt, 1983). Such separated cell populations are called compartments and the boundary is called the compartment boundary. The compartment boundaries play a crucial role in the animal development. In *Drosophila,* the wing imaginal disc is divided into two such compartments; the anterior (A) and the posterior (P) compartment. The selector gene *engrailed (en)* determines the P-compartment identity. The cells that do not express *en* take up the 'A-identity'. *en* induces the expression of a short-range signaling molecule, Hedgehog (Hh), in the P-compartment. Hh travels into the A-compartment to activate the signaling pathway. Due to the short-range effect, the signaling activity is prominently high in a stripe along the A-P boundary.

The Hh pathway is transduced by the membrane-bound, cytoplasmic and nuclear components such as Smoothened (Smo) and Cubitus interruptus (Ci). Smo is a seven-pass transmembrane protein required for the transmission of the Hh signal. Ci is the transcription factor that regulates the expression of the target genes. One of the major Hh target genes is *decapentaplegic* (*dpp*), a transforming growth factor β (TGFβ) superfamily member. Dpp is a long-range signaling molecule that regulates the genes required for growth and patterning of the wing in a concentration dependent manner (Leucit et. al., 1996). *dpp* is expressed in a narrow stripe at the boundary. This expression domain serves as a positional reference point for the patterning of the entire wing (Méthot and Basler, 1999). Therefore, the boundary has to be maintained straight to achieve the correct patterning of the wing.

The boundary is maintained by a continuous cell segregation process that counteracts the intermingling and rearrangements occurring during growth of the tissue. This segregation process was revealed by an observation of the clones deficient in an Hh pathway component, such as *smo*. Such clones generated in the A-compartment are sorted out

from the wild type surrounding into the P-compartment (Rodriguez and Basler, 1997). Thus, the Hh pathway plays an essential role in the cell segregation in order to maintain a straight boundary.

What is the mechanism that maintains a straight boundary downstream of the Hh pathway? One of the widely accepted models to explain this is the "differential cell adhesion model" (Dahmann and Basler, 1999; Schilling et al., 2011; Steinberg, 1955). This model is based on the assumption that there must be a difference in the strength of cell-cell adhesion between the two compartmental cell populations. This model led to a hypothesis that a putative compartment-specific cell adhesion molecule plays a role in generating differential affinities. Despite continuous efforts, such cell adhesion molecules have not been identified in more than a decade. Thus, whether a change in cell adhesion properties alone is sufficient for cell segregation, remains open.

In general, cell-cell adhesion is influenced by the status of the adhesion complex and contractions of the actin-myosin network. Recent studies have highlighted the latter mechanism, in which increased cell bond tension along the boundary is necessary for the straight boundary (Aliee et al., 2012; Landsberg et al., 2009; Monier et al., 2010). This provided the physical/mechanical aspect of the boundary. However, there remains a huge gap between the Hh signaling and the mechanical activities. Finding molecules that link the two activities will definitely help to better understand how the boundary is maintained.

A possible reason, why such a molecule remains unidentified, could be the lack of a suitable technique. Observation of the clones lacking a compartment identity has been a common/usual method for the study of the compartmental boundary. In the experiments, the clones were randomly generated everywhere in the wing disc. The segregation defect can be observed exclusively at the boundary. The clones showing a sorting behavior must be confirmed by examining the origin (e.g. immunostaining for a compartmental marker gene expression). Thus the number of discs showing a segregation-defect was quite

limited and a huge number of the discs need to be examined to obtain a suitable clone. Even after the selection, how the clones had moved into the opposite compartment is very difficult or impossible to analyze. In fact, no report has described segregation processes in live imaging so far. These technical hurdles have prevented an efficient genetic screening for the molecules as well as conducting detailed analysis of the segregation processes.

Here, we describe a novel technique that permits a compartment-specific clone generation in wing disc at a specific time point (Figure 2.3.1a). We name the technique, Compartment Specific Clone Generation (CSCG) technique. The technique uses a combination of genetic tools, *Gal4*/*UAS* (Brand and Perrimon, 1993), *lexA*/*lexO* (Lai and Lee, 2006), *Flp*/*FRT* (Pignoni and Zipursky, 1997; Struhl and Basler, 1993) and $Gal80^{ts}$ (McGuire et al., 2003). CSCG technique allows a detailed analysis of the segregation process and efficient screening of the genes required for generating and maintaining the boundary.

Results

The CSCG Technique

We designed the CSCG technique to generate genetically manipulable clones in a specific compartment of the wing disc, as shown in Fig (2.3.1a). This technique is a variant of the CONVERT technique in which an enhancer activity is converted into a constitutive promoter activity in a temporal manner (Yagi et al., 2010). Each compartment individually prevents the cells of one compartment from intermingling with the cells of the other compartment. Therefore we generated separate techniques for both the compartments: the A- and the P-CSCG systems.

The main difference between the A- and P-CSCG systems is the compartment-specific *LexA transactivator* (*LexA TA*) driver used. The LexA TA is a TA used in the *LexA*/*lexO* system and specifically activates expression of a *lexA operator* (*lexO*)-*effector* transgene. The A-CSCG system uses dpp^{disc}*-LG-86Fb* (*dpp-LG*) while the P-CSCG system uses *en-LHG*. The LG and LHG are Gal80-suppressible LexA TAs with different transcriptional activity: LHG has a higher activity than LG (Yagi et al., 2010). Since an *en-LexA TA* was not available, we created the *en-LHG* driver by introducing an "*en* homing" sequence (Hama et al., 1990; Kassis et al., 1992; Kwon et al., 2009) into the *pMARET-LHG* (Yagi et al., 2010).

The temperature sensitive Gal80, $tub\text{-}Gal80^{ts}$ (McGuire et al., 2003) provides a temporal control for the LexA TA activity. In the presence of $Gal80^{ts}$, the LexA TAs are inactive at 18°C and active at 29°C. When the $Gal80^{ts}$ is inactivated, these drivers activate the *lexO-flp* in respective compartments. This controllability allows choosing a specific time point and period for the clone generation.

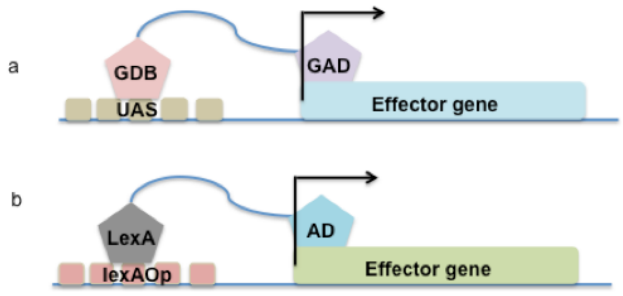


Fig. 2.1a: Binary transcriptional systems – (a) *Gal4/UAS* system: GDB = Gal4 DNA binding domain, GAD = Gal4 activation domain, UAS = Upstream activating sequence **(b)** *LexA/lexO* system: lexAOp = LexA operator, AD = a transcriptional activation domain fused to LexA.

For making the clones manipulable by the *Gal4/UAS* system, we created a *actin5C(act)>CD2,y*$^{+}$*>Gal4*^{m}. The Gal4^{m} protein contains a T860P mutation that abolishes its binding to Gal80. Thus the Gal4 is insensitive to Gal80 (Ansari, et al., 1998). Our laboratory recently generated a transgene with the *Gal4*^{m} (*dpp-Gal4*^{m}) and confirmed the functionality in fly (Of note, Gal4^{m} is slightly less active than Gal4 (data not shown)).

The Flp recombinase removes the *>CD2, y*$^{+}$*>* cassette (Struhl and Basler, 1993) from the *act>CD2,y*$^{+}$*>Gal4*^{m}. Duration of this reaction must be controlled in order to generate clones with almost the same size/age and number. The duration can be determined by how many hours the animals are cultured at 29°C.

Once the cassette is removed, the constitutive *act* promoter continuously drives the *Gal4*^{m} expression irrespective of the temperature (i.e. the Gal80^{ts} and LexA TA driver activities). The *act>Gal4*^{m} clones allow genetic manipulation with various *UAS* constructs, such as a *UAS-fluorescent* protein (e.g. *UAS-GFP*) and a *UAS-RNA*-mediated interference (*UAS-RNAi*).

In general, if a tissue-specific promoter is influenced by manipulation of a gene, the promoter-*Gal4* expression could be affected by a *UAS*-construct for the *gene*. For example, blocking the Hh signaling by a *dpp-Gal4* will influence the driver activity because *dpp* is an Hh target gene. Since the technique irreversibly converts a compartment-specific promoter activity (e.g. *dpp-LG*) to the constitutive *actin5C* promoter activity, it is free from such an artificial feedback effect.

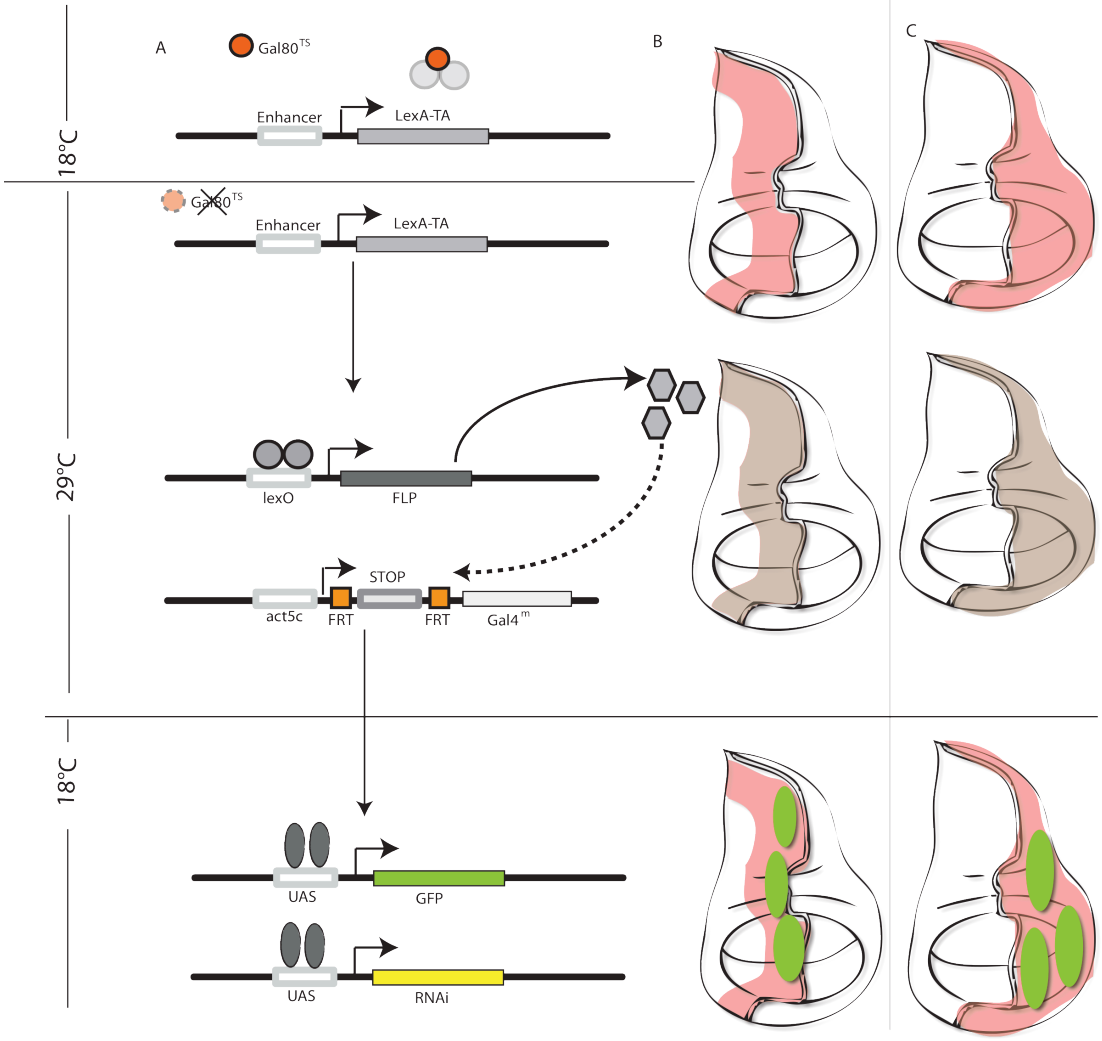


Figure 2.3.1a: The CSCG technique

(B, C) Schematics of the A- **(B)** and P-compartment **(C)** specific CSCG systems used in the technique. **(A)** The Gal80-suppressible LexA TAs are inactive at 18°C because Gal80^{ts} is active at this temperature. The system can be activated by a temperature shift from 18°C to 29°C. The LexA TAs start activating the expression of Flp recombinase. The recombinase removes the *FRT*-flanked stop cassette separating the *actin5C* promoter and *Gal4^{m}*. This reaction produces the clones expressing *Gal4^{m}*. The Gal4 activates the expression of *UAS-GFP* and a *UAS-RNAi*. The clone induction must be stopped by a temperature shift back to 18°C in order to control the frequency and to restrict the size-variability. Gal80^{ts} becomes active while the LexA TA inactive. However, the *actin5C*-driven *Gal4^{m}* clones continuously activate the *UAS-transgenes* because the Gal4^{m} is not Gal80-suppressible.

We induced compartment-specific clones by using the CGCG technique. The A- and P-CSCG systems could successfully produce A- and P-compartment specific clones, respectively. The clones were indeed manipulable with a *UAS-transgene*, as they could be labeled with the *UAS-GFP* (Fig. 2.3.1).

To characterize the system, we investigated the parameters for the clone-induction. The frequency and size of the clones (also the variation) depends on the initiation time and the period at 29°C. We determined the conditions for each system (data not shown). We found two things that the users should note. First, in the A-CSCG system, the clone generation occurs prominently at the hinge region of the disc, presumably because this is the region with the highest activity of the dpp^{disc} promoter activity at this region. Second, because it is less active at 18°C, the Gal4 activity is not at its maximum in the system (Duffy, 2002). Thus the induction time for a *UAS-transgene* should be as long as possible.

In summary, we established the two compartment-specific clone generation systems usable in various experiments.

Reproducing the Sorting Phenotype

A loss of function mutant of *smo* is known to display a sorting phenotype if the clones locate near to the boundary in the A-compartment (Rodriguez and Basler, 1997). We applied the CSCG systems to reproduce the sorting phenomenon in both the A and P compartments. To induce the sorting phenomenon with the A-CSCG system, we chose a *UAS-smo^{RNAi}* line. We confirmed the functionality of the *RNAi* by using an anti-Smo antibody. The *UAS-smo^{RNAi}* significantly decreased the levels of Smo protein in the clones, after ~80 hrs of Gal4-activation (Figure 2.3.3). As expected, the *UAS-smo^{RNAi}* clones frequently showed a sorting phenotype (~70% of the discs). Such clones invaded into the P-compartment and caused deformation at the boundary (Figure 2.3.2), consistent with the behaviors of the *smo* mutant clones (Rodriguez and Basler, 1997). We compared the frequency of spotting a clone sorting with the A-CSCG system and the conventional Gal4 flip-out system. We could more frequently find a sorting event with the A-CSCG system (70% of the total number of discs tested) as compared to the Gal4 flip-out system (50% of the total discs) (Supplementary S2).

Next we investigated if the clones exhibited reduced Hh pathway activity. For this purpose, we checked the Patched (Ptc) expression levels by using an anti Ptc antibody. Ptc is an Hh target gene. The Ptc protein levels were significantly reduced in the clones and the Ptc domain or the Hh pathway active area shifted to the anterior side of the clones (Supplementary S3).

The cells attached to the boundary have a few characteristics. The cells, in either A- or P-compartment, adjacent to the boundary (A1 and P1, respectively) display enlarged apical cross-section area compared to the cells present farther away from the boundary (Landsberg et al., 2009). The cell bonds along the compartment boundary have a 2.5-fold higher tension than those in the remaining tissue. To examine if the A-CSCG system is usable for the morphological analysis of the boundary cells, we measured the apical cell surface of the *smo^{RNAi}* cells. As expected, the *smo^{RNAi}* clones have A1 cells with slightly

smaller apical surface than the wild type cells (Figure 2.3.8 & Figure 2.3.9). Interestingly, although the *smo*^{RNAi} reduced the A1 cell surface area, the P1 cells facing the manipulated A1 cell, show the indistinguishable surface area size as the other P1 cells.

In order to find out whether the apical constriction in *smo* deficient cells is followed by apoptosis, we generated *smo* mutant clones by the MARCM technique. It has been reported that the *smo* mutant clones near the boundary get rounded up (Schilling et al., 2011). We assumed that the *smo* mutant being stronger than the *smo*^{RNAi} should manifest apoptosis more clearly, if any. However we could not spot any apoptotic cells (TUNEL positive) in the clones (Figure 2.3.7).

Together, these results indicate that the A-CSCG system reproduces the sorting phenomenon for the A-compartment clones in which the Hh pathway is impaired.

The P-CSCG System Produces Clones With Cells That Exhibit Mild Loss of P-identity

We induced clones that lost the P-compartment identity with the P-CSCG system (Figure 2.3.1). For this purpose, we used a *UAS-RNAi* line for both *en* and *invected* (*inv*). *inv* is an *en* paralogue and with *en* cooperatively determines the P-identity. Double mutant clones for *en/inv* (Gustavson et al., 1996) have been used for studies of the compartment boundary. The randomly generated mutant clones are known to display a phenotype in which they sort with the A cells.

With the P-CSCG system, we induced *en/inv*^{RNAi} clones by first maintaining the larvae at 18°C for ~80hrs AEL, then incubating them at 29°C for 12-24 hrs, again putting them back at 18°C for ~80 hrs and finally dissecting them. The En protein levels were slightly reduced under these conditions (Figure 2.3.3). However, we could not find the expected sorting behavior – the *en/inv*^{RNAi} clones didn't seem to invade into the A-compartment.

As another method to induce a sorting phenotype in the P-CSGC system, we used *UAS-Ci*^{act}. The Ci^{act} is an activator form of Ci (see thesis main introduction). Unlike the *en/inv*^{RNAi}, as expected the *UAS-ci*^{act} overexpressing clones invaded into the A-compartment (Figure 2.3.4). One disadvantage of this manipulation is the clone morphology. Since *Ci*^{act} also shows a growth-promoting activity in the tissue, the clones were always bigger and showed a rounded-up shape.

Taken together, we demonstrated that the P-CSCG system allows producing the phenotype of the clones losing their compartment-identity.

The A-CSCG System as a Tool for Genetic Screening for a Gene Playing a Role in the Boundary Maintenance

To check the suitability of the A-CSCG system as a tool for screening for the genes required in the boundary maintenance, we conducted a pilot screen with the A-CSCG system.

A former Ph.D. student in the lab, Dr. N. Simigdala, conducted a microarray analysis of genes that are differentially expressed between the A- and P-compartment (For details, see Dr. N. Simigdala's thesis). Based on the gene ontology analysis, we chose 8 candidates to be tested (Table 1). The criteria for the selection of these candidates were: 'putative Hh targets', 'membrane-bound proteins', 'cell adhesion molecules', besides, their higher expression in the A-compartment than the P-compartment. To inspect the role in the A-compartment identity, we used *RNAi* lines of the chosen candidates. If we observed a sorting phenotype upon depletion of the candidate, such a gene might be involved in the Hh signaling or regulating the actin-myosin network.

We induced and analyzed the clones of each candidate in the A-CSCG system in the same conditions as the experiment where the *smo RNAi* could cause the sorting phenotype. However, none of the candidates showed detectable sorting phenotype (Supplementary S1).

While one trivial reason might be that the genes have no effect, another might be that the system is insufficiently sensitive. Some reasons for this could be that the knockdown was not sufficient to induce a reduction of function phenotypes. This might be caused by less than maximum activity of the Gal4 or knocking down of the candidate. Because, even on knocking down *smo*, a core component of the pathway, the penetrance of the sorting phenotype doesn't seem to be high, compared to the *smo* mutant clones (Rodriguez and Basler, 1997). Besides, we actually encountered a difficulty to observe the boundary – anti En antibody staining is not so clear. Therefore, we attempted to refine the system to

be more sensitive for the sorting phenotype. For this purpose, we reduced the endogenous activity of *smo*, by using a loss of function mutant, smo^{3} (Nusslein-Volhard et al., 1984). In addition, we used an *en-lacZ* allele (Hama et al., 1990) to demarcate the *P*-compartment in the A-CSCG system. This *en-lacZ* does not show the Blair effect (Blair, 1992). In smo^{3} heterozygous background, the A-CSCG system could produce more efficient sorting event for the smo^{RNAi} clones, than the *smo* wild type background. Moreover, use of the *en-lacZ*, enables a more clear visualization of the border.

Success of the refinement prompted us to sensitize the P-CSCG system by reducing the endogenous *en/inv* activity. For this purpose we used en^{E}, a *en/inv* double null mutant (Morata G and Lawrence PA, 1975). In en^{E} heterozygous background, the P-CSCG system could produce the en/inv^{RNAi} clones more frequently attached to the boundary in comparison with the wild type background and the boundary was disturbed (Figure 2.3.6). However, we could not observe a clear sorting event even in this sensitized background.

Together, we could sensitize the systems to be suitable for the genetic screen of a gene required for the maintenance of the boundary.

Discussion

Using the CSCG technique clones can be generated in a compartment specific manner. The temporal control provided by the system allows choosing a specific time point to generate a clone. The *Gal4*^{m} expression in the clone provides the freedom to use a *UAS*-based, *RNAi* or overexpression library. The *Gal4*^{m} carries a mutation that leaves the transgene expression unaffected of the temperature change (29°C to 18°C shift back). In addition, converting a dpp-LexA TA driver into a constitutively active Gal4^{m} driver results in a stable transgene expression. The CSCG technique, by providing a means of generating clones abutting the boundary, allows analyzing the role of Hh signaling in the border-cell specific morphology.

Although the CSCG technique could produce a sorting phenotype, the persistency and the strength appeared to be low, compared with smo^{3} mutant. Therefore, we sensitized the systems by reducing the endogenous activity of the Hh pathway. There are two possible reasons why the apparent knockdown efficiency was low. First, the Gal4 activity of the system was lower compared to the conventional Gal4 flip out system. The system needs to decrease the culture temperature at 18°C during induction of the Gal4/UAS system. The Gal4 is temperature sensitive and the activity is lower at 18°C than at 25°C (Duffy, 2002). To increase the sorting phenotype, it would be better to use a Gal4 derivative with a more active transactivation domain, such as Gal4::VP16 (Sadowski et.al., 1988) or the progesterone-inducible Gal4::PR (Osterwalder et al., 2001). Second, efficacy of the RNAi for the target was low. We observed the *en/inv*^{RNAi} did not significantly reduce the endogenous En protein. The En and Inv protein might be stable proteins and therefore the RNAi did not work efficiently in these conditions. Use of *UAS-Dicer2* coexpression may potentiate the efficacy of the RNAi.

The CSCG technique is already a sophisticated system with high manipulability. However, if the Gal4::PR is used, instead of Gal4^{m}, the modified system will provide one

more parameter that can be tuned – the inducibility and the dose dependency of the Gal4 activity for the progesterone-derivative RU486. This would permits induction of the Gal4 activity after the clone grew to a certain size. Such a system produces an insight into whether the clones are collectively sorted out into the opposite compartment.

We attempted to find a novel gene required for the maintenance of the boundary. The setup of the pilot screen was such that we could find factors that play a major role in the process. However, downstream of the Hh pathway might have diversified and thus multiple target genes may work cooperatively in the process. To identify such genes with minor contribution it would be useful to conduct a dominant suppressor/enhancer screening accompanied with the smo^{RNAi} sorting phenotype. Since one copy loss of the *smo* (smo^{3} heterozygous) enhanced the phenotype, it is likely to work as well.

Here we established the CSCG technique, aimed for investigating the molecular mechanism for the maintenance of the A-P boundary. The concept can also be applied to investigating the D-V boundary of the wing discs, where the Notch signaling pathway plays a central role. Such system needs a D or a V compartment-specific LexA TA driver (e.g. a D compartment-specific *apterous*-LexA TA driver). The Cas9/CRISPR technique (Ran et al., 2013) provides a way to generate such driver lines.

The CSCG technique can facilitate studies of other biological phenomena. For example, the P-CSCG system allows precise analysis of cell competition and regeneration. In such experiments, a user observes the event of cell competition/regeneration in comparison with the control, the A compartment. In another embodiment a A-CSCG variant would permit specific and clonal manipulation of the morphogenetic furrow in the eye disc, since *dpp* is expressed in the eye disc furrow (Corrigall et al., 2007; Heberlein et al., 1993).

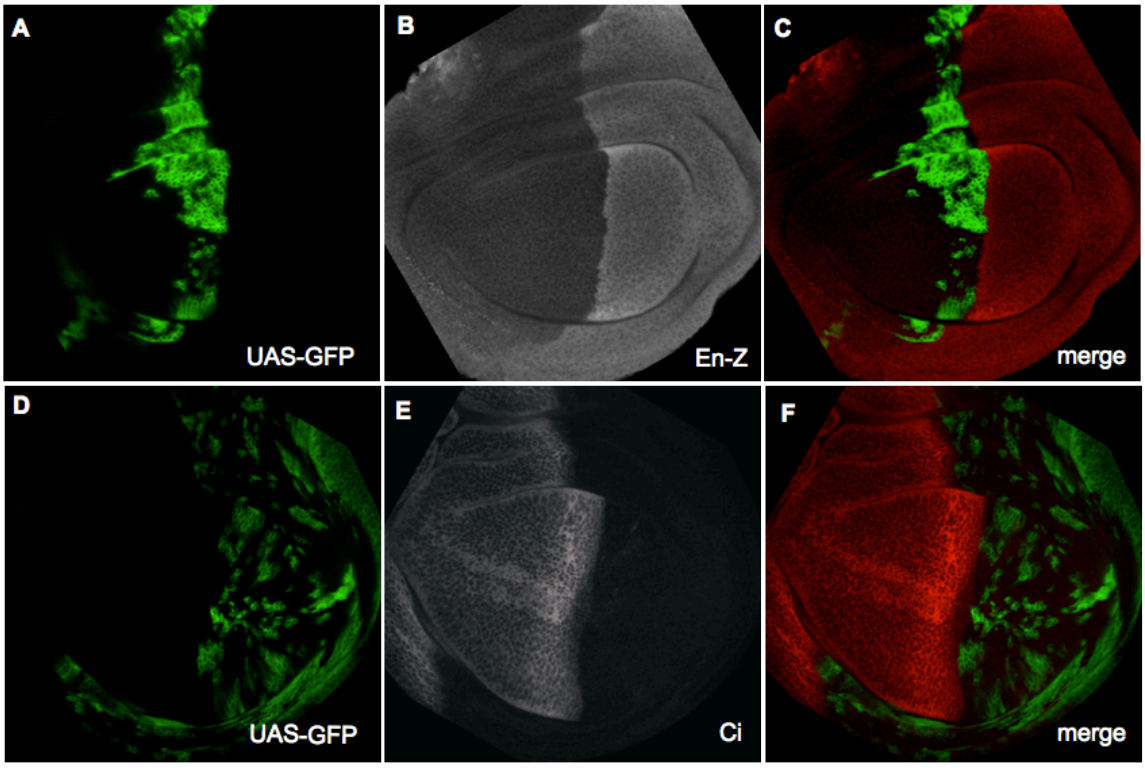


Figure 2.3.1: Each CSCG system generates clones in its specific compartment

(A-C) GFP-marked clones are specifically generated in the anterior compartment, shown by En staining (B) demarcate the posterior compartment. **(D-F)** GFP marked clones (D) are specifically generated in the posterior compartment, shown by Ci staining (E) demarcate the anterior compartment.

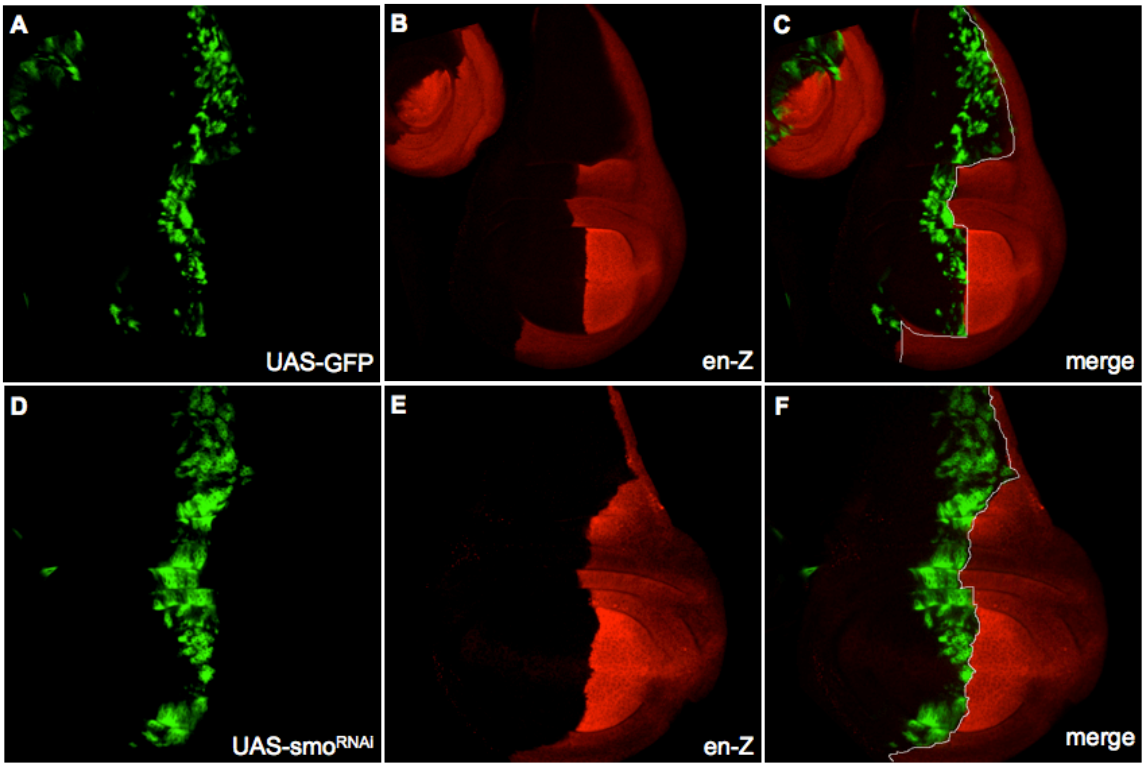


Figure 2.3.2: Sorting induction by A-CSCG system

The GFP marked clones expressing *UAS-smo*^{RNAi} displays sorting behaviors. **(A)** invading the posterior compartment as shown by *en-lacZ* (en-Z; anti βGal antibody) staining **(B)** marking the irregular boundary.

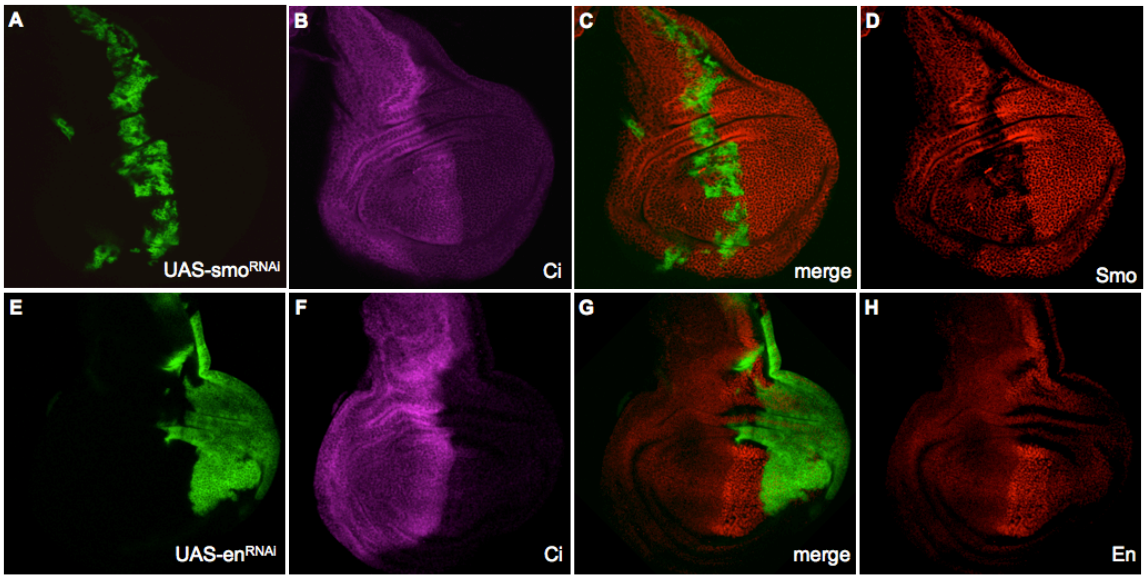


Figure 2.3.3: RNAi efficiency

(A-D) *smo*^{RNAi} expressing GFP marked clones (A) show decreased Smo levels (D). **(E-H)** *en*^{RNAi} expressing GFP marked clones (E) show only slight decrease in En levels (H).

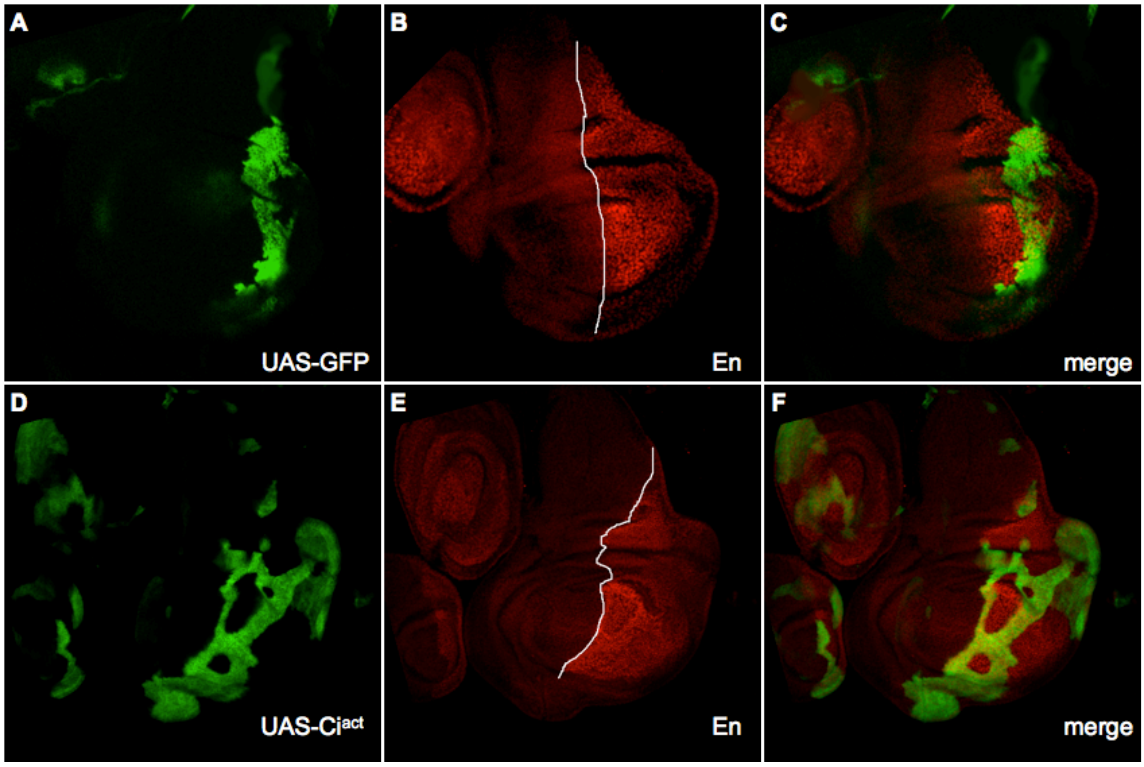


Figure 2.3.4: *UAS-Ci^{act}* overexpressing clones in P-compartment

Control clones in P-compartment **(A,B &C)**

UAS-Ci^{act} expressing clones **(D)** invade the anterior compartment **(E&F)**.

No.	Gene	A vs. P ratio	Biological/Molecular function
1	CG8967 (off-track)	2.36	cell adhesion, molecule binding, receptor activity axon guidance
2	CG32425	3.37	Nothing known
3	CG8965	5.7	signal transduction
4	CG4451 (hep sul 6-0 transferase)	8	wing margin development, wnt/dpp signaling
5	CG155529	9.5	Nothing known
6	CG12768	15.46	Nothing known
7	CG8503	18.35	histone deacetylase binding, negative regulation of gene expression
8	CG4398	18.93	Nothing known
9	CG9593	22.97	receptor binding, signal transduction

Table 1: List of genes used in the A-CSCG system screen for those that play a role in cell sorting.

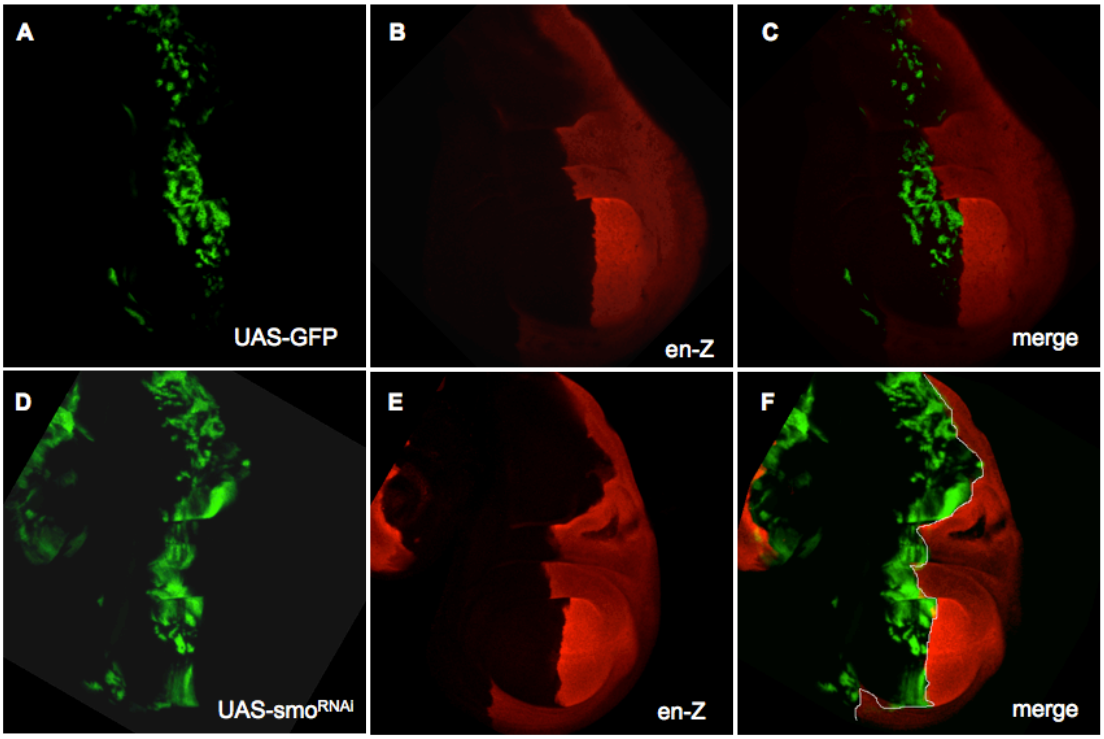


Figure 2.3.5: Sensitized background enhances sorting phenotype

(A-C) wild type GFP expressing clones in *smo*^{3} background **(D-E)** in *smo*^{3} sensitized background *smo*^{RNAi} clones penetrance more into the P-compartment and the level of the boundary disturbance increases.

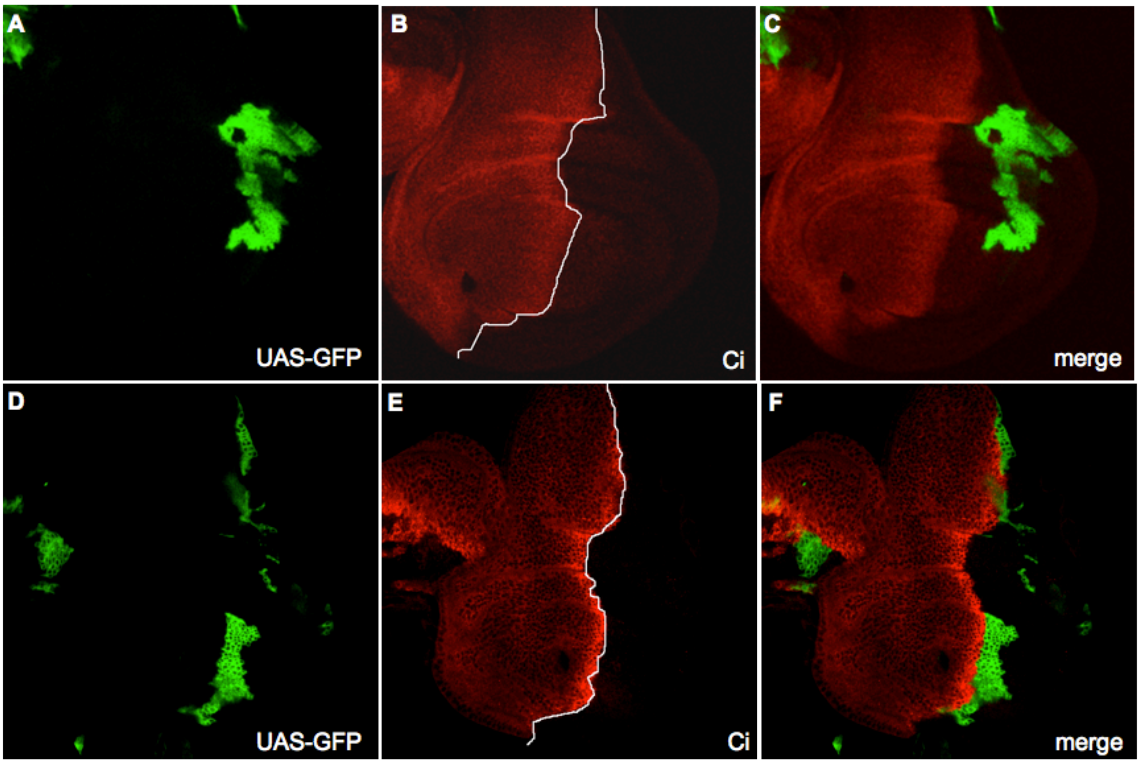


Figure 2.3.6: Sensitized background enhances sorting phenotype

(A-C) wild type GFP expressing clones in en^{E} background **(D-E)** in en^{E} sensitized background en/inv^{RNAi} clones often stuck to the boundary and the level of boundary disturbance also seems to be increased.

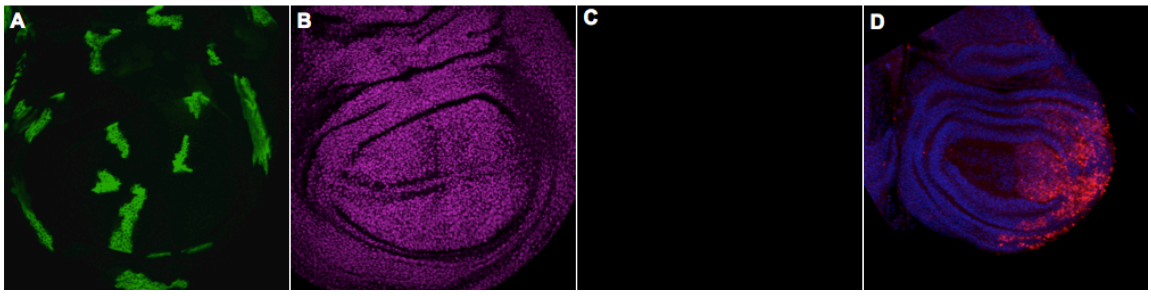


Figure 2.3.7: Test of apoptotic cells in *smo* mutant clones near the boundary

(A) by MARCM technique randomly generated *smo* mutant clones (green) **(C)** no TUNEL positive cells **(D)** positive control; TUNEL positive *UAS-hid* clones generated only in the posterior compartment.

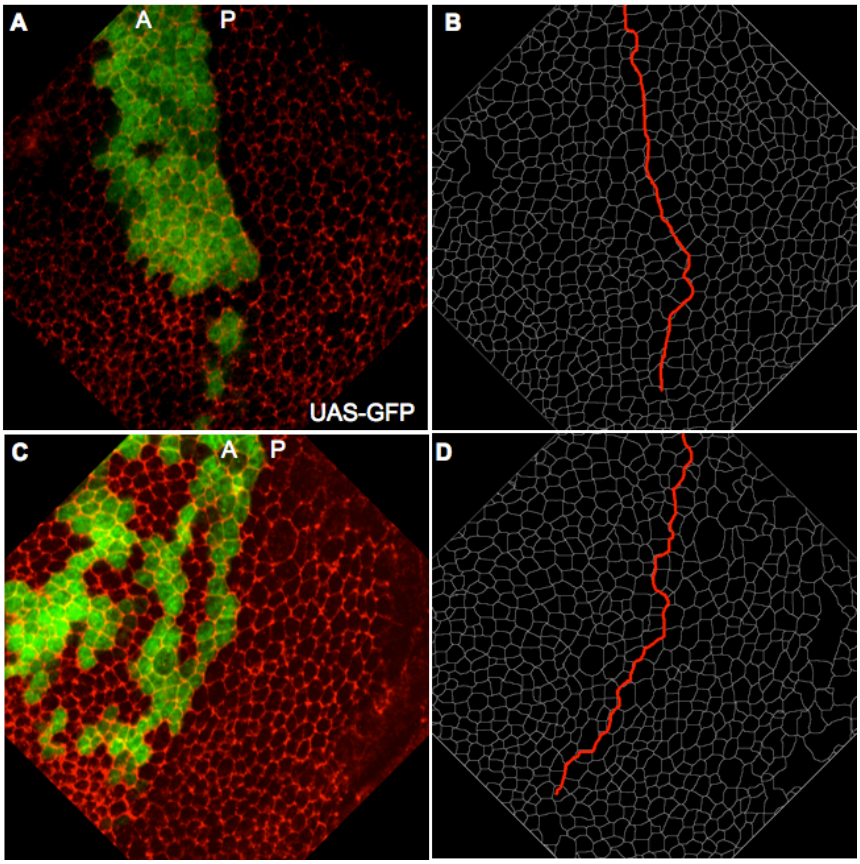


Figure 2.3.8: Manipulation of border cells by A-CSCG system

(A-B) GFP marked wild type clones abutting the A-P boundary. **(C-D)** GFP marked smo^{RNAi} overexpressing clones abutting the boundary.

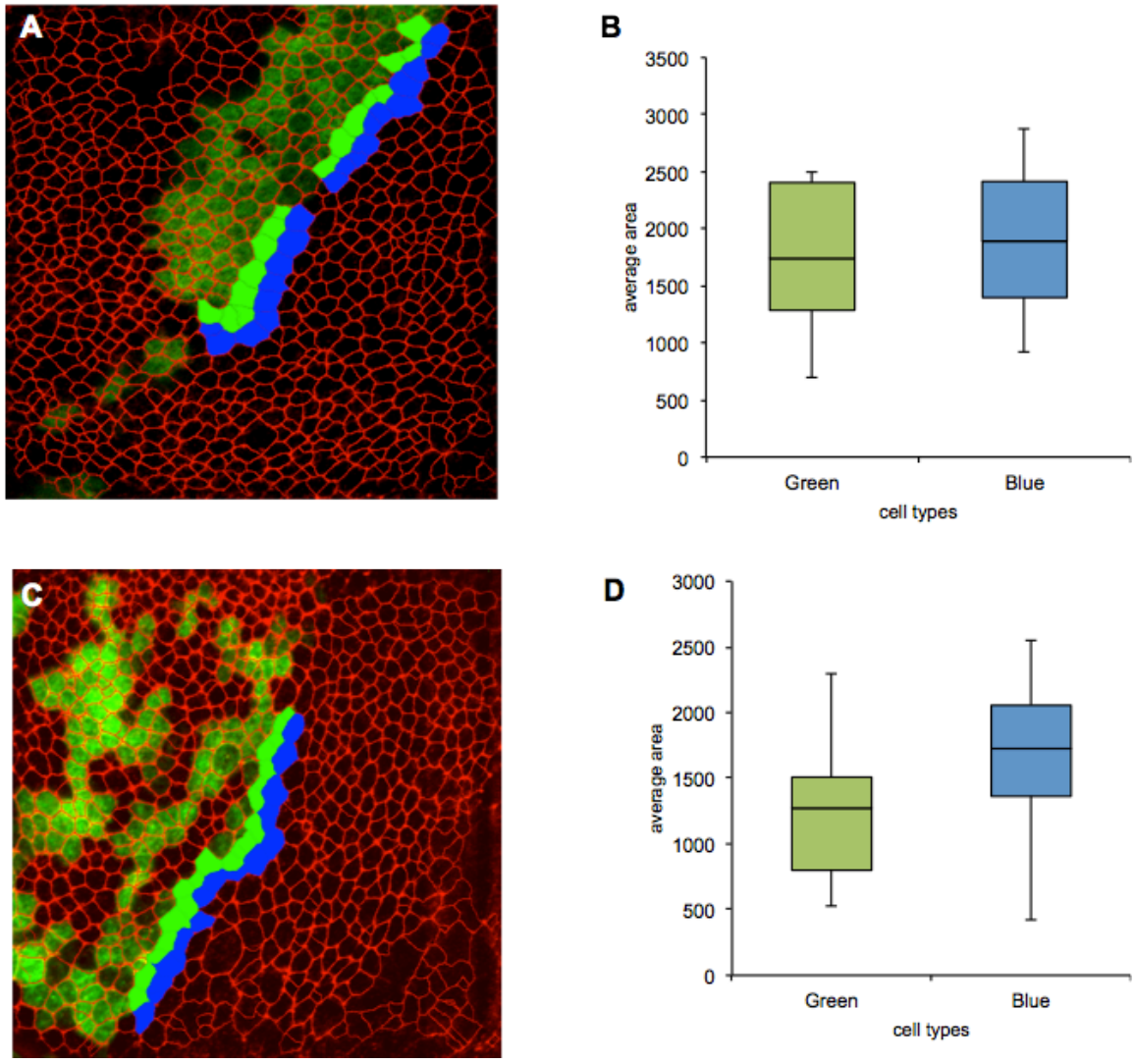


Figure 2.3.9: Change in apical surface area of the manipulated border cells

(A&C) parts of the clone were taken for analysis that is in the dorsal half of the wing disc pouch, with a similar distance to the hinge region and the dorsal-ventral compartment boundary. **(B&D)** quantification of the apical area of A1 and P1 cells in both mutant and wild type clone.

Materials and Methods

Drosophila Stock List

The following Drosophila stocks were used in this thesis:

yw P{≥Gal4,w$^+$}^{mr25}	from R. Yagi
yw; Δ2-3 TM3 Sb/ CxD	from D. Brunner
yw, UAS-GFPy$^+$	
yw;lexO-flp-22A, tubGal80^{ts} / CyO; MKRS/TM6B	from R. Yagi
yw; lexO-flp-22A/CyO; dppLG/TM6B	from R. Yagi
yw UAS-CD8::GFP hsp-flp; Sp/CyO; MKRS/TM6B	from R. Yagi
yw UAS-CD8::GFP; Sp/CyO; MKRS/TM6B	from R. Yagi
yw hsflp; FRT40 smo^3/CyO	(N. Methot & K. Basler 1999)
yw hsp-flp; smo^3 FRT39 enZ/CyO	(N. Methot & K. Basler 1999)
yw UAS-mCD8::GFP hsp-flp; ; tub-Gal80	from Ernst Hafen
FRT40/SM5^TM6B; tub-Ga4/SM5^TM6B	
yw; Sp/CyO; tub-Gal80^{ts} / TM6B	from R. Yagi
yw; lexO-flp-22A/ CyO; TM2/TM6B	from R. Yagi
yw hsp-flp; en^E FRT42/CyO y$^+$	(N. Methot & K. Basler 1999)
yw hsp-flp; act>CD2>Gal4, UAS-GFP	(M. Vegh & K. Basler 2003)
yw hsp-flp; en-lacZ/CyO	(N. Methot & K. Basler 1999)
smo-RNAi	from G. Reim
en/inv-RNAi VDRC 35697	from VDRC
CG4398-RNAi VDRC 104199	from VDRC
CG8503-RNAi VDRC 24113	from VDRC
CG8567-RNAi VDRC 104881	from VDRC
CG9593-RNAi VDRC 24165	from VDRC

CG8965-RNAi VDRC 23661	from VDRC
CG4451-RNAi VDRC 42658	from VDRC
CG32425-RNAi VDRC 41143	from VDRC

A and P-CSCG Systems Were Generated by the Following Fly Crosses:

CSCG system needs the following components: (i) *tub-Gal80^{ts}*, (ii) compartment-specific LexA-transactivator (LexA TA) driver, (iii) *lexO-flp-22A*, (iv) *act>CD2,y^{+}>Gal4^{m}* and (v) *UAS-CD8::GFP*. We chose *dpp-LG-86Fb* (Yagi et al., 2010) and *en-LHG* (for cloning details see 2.2) for the A and P compartment-specific LexA-transactivator (LexA TA) drivers respectively.

A-CSCG system was generated by the recombination of transgenes *lexO-flp-22A* and *tub-Gal80^{ts}* onto the same 2nd chromosome; and *dpp-LG-86Fb* and *act>CD2,y^{+}>Gal4^{m}* onto the same 3rd chromosome. *UAS-CD8::GFP* on X chromosome was included to facilitate testing *UAS*-driven transgenes. For P-CSCG system, recombined transgenes were *en-LHG* and *lexO-flp-22A* onto the same 2nd chromosome; and *tub-Gal80^{ts}* and *act>CD2,y^{+}>Gal4^{m}* onto the same 3rd chromosome with *UAS-CD8::GFP* on X-chromosome.

Experimental Animals Contain the Following Genotype:

Figure 2.3.1:
UAS-CD8::GFP; lexO-flp-22A, tubGal80^{ts}/CyO; dpp-LG-86Fb, act>CD2, y^{+}>Gal4^{m}/TM6B (A-CSCG system) crossed to *ywhsp-flp; en-lacZ/CyO*
UAS-CD8::GFP; lexO-flp-22A, en-LHG/CyO; tubGal80^{ts}, act>CD2,y^{+}>Gal4^{m}/TM6B crossed to *yw*

Figure 2.3.2, 3, 8 & 9:

UAS-CD8::GFP; lexO-flp-22A, tubGal80^{ts}/CyO; dpp-LG-86Fb, act>CD2, y^{+}>Gal4^{m}/TM6B (A-CSCG system) crossed to *yw; en-lacZ/CyO; UAS-smo^{RNAi}*

UAS-CD8::GFP; lexO-flp-22A, en-LHG/CyO; tubGal80^{ts}, act>CD2,y^{+}>Gal4^{m}/TM6B crossed to *UAS-en/inv^{RNAi}*

Figure 2.3.4:

UAS-CD8::GFP; lexO-flp-22A, en-LHG/CyO; tubGal80^{ts}, act>CD2,y^{+}>Gal4^{m}/TM6B crossed to *UAS-Ci^{act}*

Figure 2.3.5:

A-CSCG system crossed to *yw hsp-flp; smo^{3} FRT39 enZ/CyO; UAS-smo^{RNAi}*

Figure 2.3.6:

P-CSCG system crossed to *yw hsp-flp; en^{E}FRT42/CyO y^{+}* crossed to *UAS-en/in*

Figure 2.3.7:

yw hsflp; smo^{3}FRT40/CyO

yw UAS-mCD8::GFP hsp-flp; tub-Gal80 FRT40/SM5^TM6B; tub-Ga4/SM5^TM6B

yw hsflp; en-Gal4, UAS>CD2,y^{+}>hid/CyO; MKRS/TM6B

Vectors

For cloning standard molecular biology cloning methods were applied.

To generate *en-LHG-IG, pIG048* and *pGalW* are digested with EcoRV and NotI+blunted respectively and ligated together that gives *pIG049*. AvrII restriction of *pIG042* and *pIG049* gave *pIG045*. Finally NotI digestion of *pLattBy^{+}* and *pIG045* gave *en-LHG-IG (pIG050)*. For more details see plasmid list.

To generate *act>CD2,y+>Gal4m* flip out construct with less sensitive FRT site, two shortened FRT flanked CD2, y^+ stop cassette, was cloned in a plasmid that contained *actin5C* promoter followed by mutated Gal4, $Gal4^m$ and an *attB* site for phage mediated transgenesis. The construct was integrated at the landing site *ZH-86Fb* (Bischof et al., 2007).

To generate N-terminal flu tagged Ci^{act} overexpression construct, the CDS of Ci^{act} (Méthot and Basler, 2000) and flu tag were cloned in *pUASattB* vector. By the *PhiC31* transgenesis system it was integrated on the landing site *ZH-86Fb* (Bischof et al., 2007).

A detailed list of primers and constructs used is available upon request.

Clone Induction

In all experiments with the CSCG system, clones were generated by shifting the larvae from 18°C to 29°C for 12-24 hrs at 3-4 days after egg laying (AEL). Larvae were kept at 18°C for another 3-4 days before they were dissected.

For quantification of the sorting frequency by the FLP/FRT system and MARCM clones were generated by heat-shocking the larvae for 30 mins at 37°C at 72 hrs (+-12hrs) after egg laying (AEL). The larvae were dissected 2-3 days after clone induction.

Immunohistochemistry and Image Processing

Immunostainings were performed using standard protocols. Images were taken with Zeiss Lsm710 confocal microscope using 25x and 63x oil objectives and images were analyzed with the imageJ software. Maximum Z projections were performed for the individual micrographs. Discs were correctly oriented and processed in Adobe Photoshop.

Cell morphology was analyzed using Packing Analyzer (B. Aigouy et al., 2010) and a software tool (D.M. Heller et. al., 2004, manuscript in preparation). Parts of the clone were taken for analysis that is in the dorsal half of the wing disc pouch, with a similar distance to the hinge region and the dorsal-ventral compartment boundary.

The following antibodies were used: mouse anti-Engrailed (1:200; DSHB), chicken anti-β galactosidase (1:200, ICL), mouse anti-Smoothened (1:200; DSHB), mouse anti-Patched (1:200; DSHB), rat anti-Cubitus Interruptus (1:200; DSHB). Secondary antibodies used were Alexa Fluor 594 goat anti-mouse, Alexa Fluor 594 goat anti-chicken, Alexa Fluor 594 goat anti-rat, all from Molecular Probes, 1:400.

Plasmid List

pIG048	*loxP-Gal4-attP*+TOPO	PCR(*pMARET-G4**; IND5, IND6)
pIG042	*en* homing+TOPO	PCR(BAC; IND1, IND2)
pIG049	*loxP-Gal4-attP+pGalW*	*pIG048*xEcoRV into *pGalW****xNotI/blunted
pIG045	*en* homing+*pIG049*	*pIG042*xAvrII into *pIG049*xAvrII
pIG050	*LHG,y*$^+$ + *pIG045*	*pLattBy*$^+$*-LHG***xNotI into *pIG045*xNotI
pIG060	*>CD2,y*$^+$*>* + *pA5CattB-G4*^{TP}	*FC17x*Acc65I into *pA5CattB-G4*^{TP} x Acc65I

* and ** (Yagi et al., 2010).

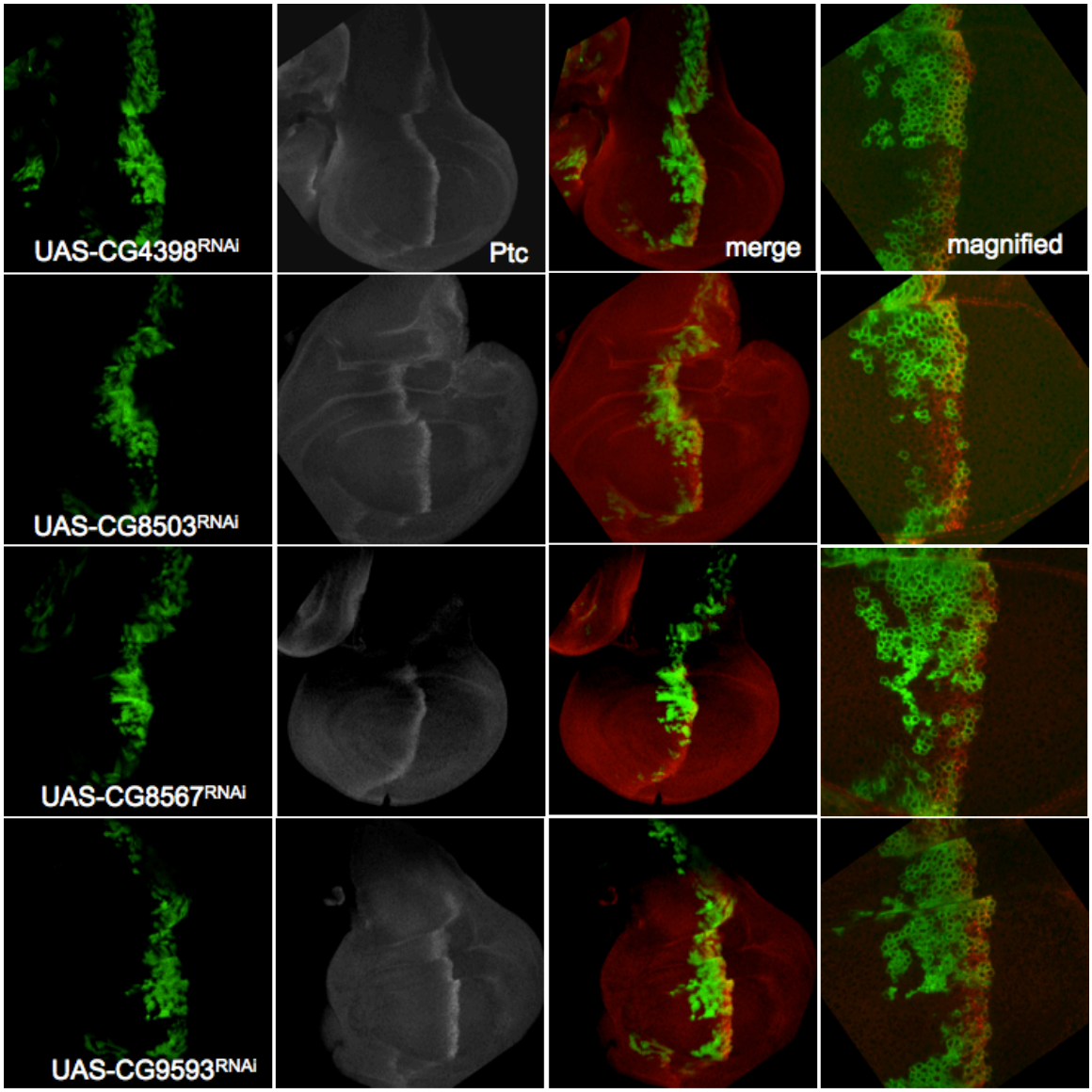
*** (Gerlitz et al., 2002).

Primer List

Primer ID	Primer seq.	Comments
IND-1	AAACCTAGGGAATTCCGTTGATATGAT	BAC_en_AvrII_FP
IND-2	AAACCTAGGGCGAGTTGCGATTTAGCACCAAG	BAC_en_AvrII_RP
IND-3	AAACCTAGGCTTGGTGCTAAATCGCAACTCGC	
IND-5	AAAGGATCCCTTACGCTAACCGGTGG	
IND-6	AAAGGATCCGGGCGCTCCTCGGTG	
IND-7	GGAATGGAACGGTGTGGAAG	enP_27330_1FP
IND-8	CGCCACCACTCACACTCCC	enP_27909_1RP
IND-9	GTTTGGGCGTTAGTTTGTT	enP_27857_2FP
IND-10	CGAATATCGATTTGCCAG	enP_28306_2RP
IND-11	CAATGCCAAATGGCGTTCG	enP_28228_3FP
IND-12	TCCCTCTCGCTCTCACTCT	enP_28929_3RP
IND-13	GGCTCAGTGTCAAGTGACCCAG	enP_4FP
IND-16	GAGAGGAAAGGTTGTGTGCGG	5'P_FP
IND-17	CAACAAGCAAACGTGCACTG	5'P_RP
IND-18	CCTTTCACTCGCACTTATTGC	3'P_FP
IND-19	GACAGCGATATGATTGTTG	3'P_RP
IND-20	CCTCTCAACAAGCAAACGTGCACTG	5'P_BfaI_RP
IND-21	GCCTCCTGAAAGATGAAGCTACTG	5'P_BfaI_FP
IND-22	CGCACTTATTGCAAGCATACG	3'P_BfaI_FP
IND-23	GCTTGTCGGCGTCATCAACTCC	3'P_BfaI_RP
IND-24	CCTCTTTGTCGGTCAAGTGTCGATCC	CG42669_RP_8515
IND-25	CCTCTTTTCATCGACAGCTGCGTTC	CG42669_RP_8955
IND-26	CCTAGGATCCTTGGAGCTCCTTCAGGA	HL FP
IND-27	CCTAGGAATTAACCCCGCAGGTCCACC	HL RP

Supplemental Data

Figure S1



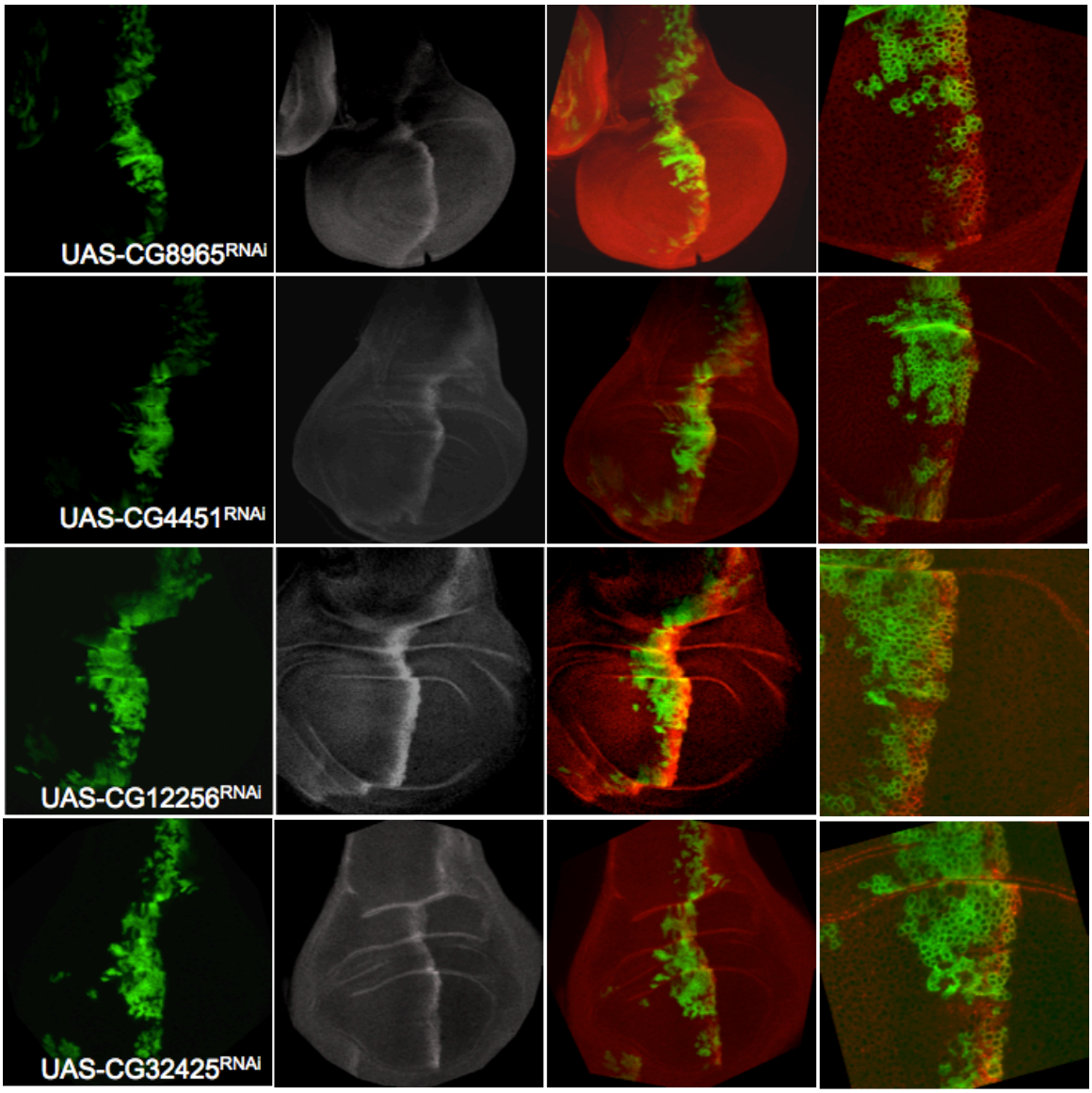


Figure S1: Screen for genes that may play a role in cell sorting

Anti-Ptc antibody staining in grey/red and *RNAi* against various molecules is expressed in the GFP marked clones. Straightness of the boundary marked by Ptc staining was carefully investigated in the pouch region (extreme right panel).

Figure S2

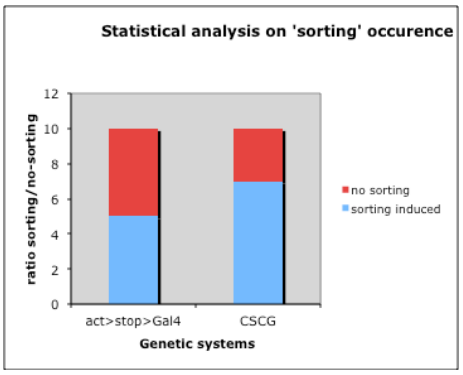


Figure S2: Comparison of *hsp-flp; act>stop>Gal4* and A-CSCG system for their ability to induce sorting

smo^{RNAi} clones were generated everywhere and in the *dpp* domain of the wing disc by *hsp-flp; act>stop>Gal4* and A-CSCG systems, respectively. In total 10 wing discs were examined for each system separately. Any irregularity in the boundary was scored as positive sorting event. Red part of the bar shows no sorting and blue shows number of discs that shows any sorting (or irregular boundary).

Figure S3

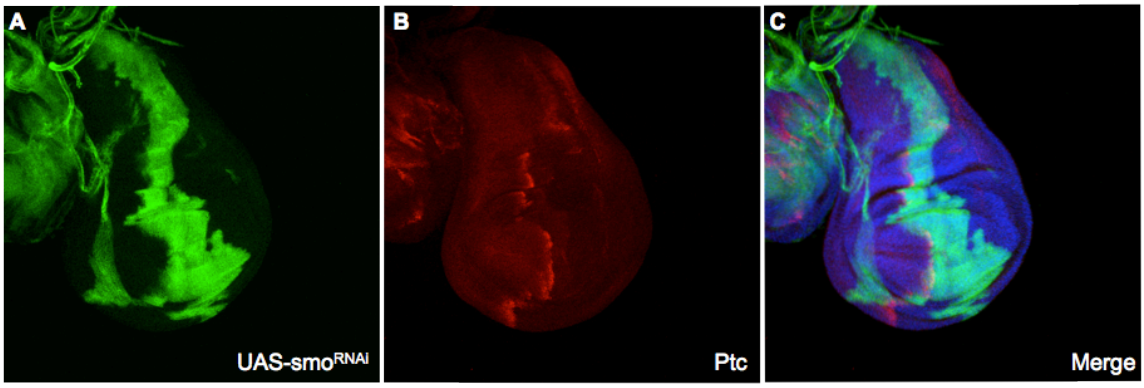


Figure S3: Hh pathway blocking efficiency:

(A) GFP marked clone* expresses *UAS-smo^{RNAi}* **(B & C)** reduced Ptc expression in the clone, Ptc expression domain shifted towards the anterior side of the *smo^{RNAi}* clone

* In this particular experiment in order to block Hh pathway in the entire *dpp* domain we used a variant of A-CSCG system, A-CSCG^{PR} system (details mentioned elsewhere). This system contains a normal flip-out cassette explained here, hence the flipping out efficiency is much higher than normal A-CSCG system and almost the entire *dpp* domain expresses act>Gal4.

Abbreviations

>	wild type *FRT* sequence
≥	*loxP* sequence
A-CSCG	A-compartment specific clone generation system
AEL	After egg laying
BAC	Bacterial Artificial Chromosome
DNA	Deoxyribonucleic acid
Dpp	Decapentaplegic
dpp^{disc}	*dpp* disc enhancer
en/En	*engrailed*/Engrailed
en-homing	*en* promoter sequences that induce homing phenomenon
Flp	Recombinase that excises DNA sequences flanked by *FRT* sites
flu	Triple HA
GAD	Gal4 activation domain
Gal4	Transcriptional activator
$Gal4^{m}$	$Gal4T^{860P}$, a Gal80 insuppressible form
Gal80	Yeast repressor for Gal4
$Gal80^{TS}$	Temperature sensitive form of Gal80
Hh	Hedgehog
LexA	E.coli transcriptional activator
lexO	*lexA* operator
LG	LexA::GAD
LHG	LexA::H::GAD
P-CSCG	P- compartment specific clone generation system
PCR	Polymerase chain reaction
RNAi	RNA interference
UAS	Upstream activating sequence
ΦC31	Integrase that mediates DNA integration in the genome

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